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FOREWORD

The purpose of this publication is to provide a readily available reference book of methods suitable for the analysis of the natural constituents, additives, and biological and environmental residues that may occur in meat and poultry products. In addition, the book covers policy, developed by FSIS, Science, USDA, on the use of additives, both direct and indirect, that is applicable to meat and poultry products.

The book is divided into six major parts: The first two parts cover policy on meat products, and nonmeat products. The remaining parts deal with methodology for macroanalytical, microanalytical, biological residues, and nutritional procedures. Each part is numbered and the pages within a part are numbered accordingly (i.e. part 2, pages 2-1, 2-2, 2-3, etc.). This arrangement and the loose-leaf format of the book provide a means of easily adding changes, corrections, and new procedures.

The references to the analytical procedures included in this publication are listed at the end of each method. We gratefully acknowledge the permission of the Association of Official Analytical Chemists (AOAC) for allowing the use of selected methods. This Agency accepts sole responsibility for the accuracy of transcribing and the paraphrasing thereof. If there is doubt about the accuracy of a method, the original reference should be reviewed. Terms used in this publication are defined in the same manner as the AOAC Book of Methods, 14th Edition, pages xxi-xxvi. The status of each method is indicated in "Contents" using the following letter designations:

Official—Methods that provide a basis for regulatory action without additional product analysis.

A. AOAC Official Method—The method is subjected to an extensive interlaboratory study in which five or more laboratories participate. This process provides results that establish the acceptability of the method. It is accepted as an official procedure by the AOAC.

B. Validated Method—The method is subjected to an interlaboratory study in three laboratories. The resulting data is reviewed by a peer group of government scientists. The data that results from the study is made available for review upon request.

C. Federal Register Method—Method of analysis published in the Federal Register and later incorporated into the Code of Federal Regulations.

D. Historical Official Method—Method considered to be the best available at the time of initial acceptance and continued in use over an extended period of time.

Unofficial—Methods that require additional product analysis before regulatory action is taken. They are used to determine the need for official methods in produce testing, and as a preliminary phase of official methods development.

E. Pilot Study Method—Method developed in a single laboratory, resulting in statistics on method performance. It is used to determine the need for additional testing and/or the development of official methods.

F. Screening Method—Semi-quantitative method used to quickly determine the presence or absence of compounds. Enables rapid screening of large numbers of samples in less time than would be required with official methods. Positive results at or above specified levels require further analysis by an official method.

G. Published Method—Method that has been published and has been subjected to a ruggedness test in an FSIS, Science laboratory but has not been thoroughly evaluated outside the originating laboratory. It may be used in nonrecurring analyses and requires the development of a rigorous protocol for sample analysis. Before the acceptance of analytical results for regulatory action, it is necessary to repeat the entire series of analyses using an official method.

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CHEMISTRY LABORATORY GUIDEBOOK

PART 1—MEAT PRODUCTS

1.001 Integrity of Samples

Each sample received at a laboratory for analysis must be examined upon receipt to ascertain that its condition is acceptable to insure reliable analytical results. Those received in an unsatisfactory condition are not analyzed. The sender must be notified promptly when a sample cannot be analyzed.

The supervisor should check the incoming samples and instruct the operator in the handling of unusual ones. Samples that are too small or otherwise unfit for analysis (spoiled, contaminated, etc.) should be discarded and resampling requested.

Examples of conditions that frequently make samples unsuitable for analysis are as follows:

(1) For fat, added water, or added substance in meats:

- (a) Exposure to air through rupture of immediate container.
- (b) Leakage of container.
- (c) Putrid.
- (d) Inadequate size sample less than $\frac{3}{4}$ lb.
- (e) Absorbent material in contact with sample.

(f) Sample not frozen or canned for nitrite determination, when sodium nitrate and/or potassium nitrate are declared on the label. If nitrates are not declared on the label, sample need not be frozen or canned upon receipt but must be in sound condition. Dry products which are shelf stable do not require receipt in a frozen condition.

(g) Sample not frozen or canned when submitted from maximum internal temperature determination.

(2) Spices and seasonings.

- (a) Inadequate sample less than 2 oz.
- (b) Lack of proper identification.

The laboratory will return the sample report form to the inspector with the following information:

- (1) The condition of the sample as received and the reason why it could not be analyzed.
- (2) Appropriate suggestions for improvement of resampling and shipping techniques.

1.002 Sample Discard Criteria and Preparation

1.002A Sample Discard Criteria

I. Introduction

Chemical analysis must not be performed when the official sample is unsuitable. Such samples must be discarded. Discard criteria for specific chemical residue (part 1.002A II) and food chemistry (part 1.002A III) samples are as follows:

II. Residue Samples

A. Before discarding any meat or poultry surveillance samples for specific chemical residues, contact the Regional Office to learn whether the sample must be analyzed, or may be discarded.

B. Discard any meat or poultry monitoring samples for specific chemical residues if:

1. The sample has no identification, or illegible identification.
2. The sample bag is broken or left open so that the contents are exposed.
3. The sample is judged to be spoiled or rancid. (Requires supervisory concurrence.)
4. The sample contains comingled tissue, i.e., two or more samples in contact with each other.
5. The sample is improper tissue for the desired analysis.
6. The sample size is insufficient. MPI Directive 917.1, Rev. 2, Exhibit D specifies sample size for each residue sufficient to perform screening and official tests. (See section 1.002A II A.)
7. The shipment of the sample is delayed so that the reporting of the final results cannot meet the required reporting time of three weeks from the date of *sample collection*; this includes transhipped samples.
8. The sample arrives in the laboratory without the presence of ice crystals. This applies only to residue codes 300, 500, 600, 700, and 900. Except for code 100, fatty tissues samples must be 28°F. or less.

III. Food Chemistry Samples

Discard any meat and poultry food chemistry sample if:

1. The sample has no identification or illegible identification.
2. The sample bag leaked, is leaking, or is open.
3. The sample is judged to be spoiled or rancid. (Requires concurrence of supervisor.)
4. The sample weighs less than 12 ounces (except for cures and pickles and very dry processed products which should weigh at least three ounces).
5. The sample is improperly packaged; such as report forms or absorbent paper in contact with the sample, or cooked sausage samples requiring compliance with the 30% fat limitation which are submitted in vacuum packages.
6. The sample does not contain ice crystals, as in the case of samples analyzed for their nonfat dry milk, corn syrup solids and curing accelerators content, as well as pickle solutions, and analysis of canned hams for maximum internal temperature by the phosphatase procedure. Canned hams requiring maximum internal temperature analysis are not required to arrive with ice crystals present *unless* the inspector has removed a center portion of the ham from its can and

submitted it to the laboratory in a plastic bag. In this case, that portion of the “canned” ham must arrive in the laboratory with ice crystals present.

7. The split samples are not identified with a three digit sample number.

8. The split sample is not properly prepared.

9. The cooked sausage samples requiring compliance with the 30% fat limitation do not consist of three one pound units. *Exception:* Do not discard any samples which the inspector has marked “Special”, whether they are from a small producer, or are 30-one pound samples from a resampling procedure.

1.002B Food Chemistry Sample Preparation

I. Introduction

The objective of sample preparation is to obtain a finely chopped homogeneous sample for chemical analysis that truly represents the sample the inspector submitted to the laboratory. Care must be taken when chopping and mixing the sample to include all of the liquid, fat, gelatin, and any other portions of the sample. Sample preparation should be completed expeditiously to minimize the loss of moisture due to evaporation. Prepared samples should be immediately analyzed or refrigerated. Sample discard criteria must be applied to determine acceptability of samples for chemical analyses before chopping operations begin. In addition, priorities due to the nature of analytical requests, (such as requests for nitrite, nitrate, maximum internal temperature, lactose, etc.), must be considered.

II. Equipment

A. Meat chopper with plate holes of 1/8" or less. (5/64" plate holes are optimum.)

B. Bowl choppers capable of handling 5 to 22 pound samples or a Hobart Blender Model VCM-25 or similar device capable of handling up to 50 pound samples.

C. Chopper plates and knives numbered and used in pairs.

D. Chopper knives maintained sharp: chopper plates to be replaced when worn. Grooved plates and nicked knives indicate wear.

E. Chopper worms must be matched with chopper barrels and replaced when worn. Poor chopping speed is an indication of worn chopper barrels and worms.

F. Stainless steel mixing bowls available in sufficient sizes to handle 1 to 22 pound samples.

G. Stainless steel spatulas.

H. Wooden or plastic plungers. Do not use fingers or spatulas for this purpose.

III. Sample Preparation Procedures

All sample chopping is to be performed using clean and dry equipment which is at room-temperature. Do not open more than one sample at a time before chopping. All inedible casings are to be removed prior to sample chopping.

A. Emulsified Samples—chop emulsified samples, such as frankfurters, wieners, and bologna, twice with careful mixing with a spatula between each chopping.

B. Non-emulsified Samples and Whole Cuts—chop non-emulsified samples, such as ground beef, pork sausage, bacon, salami, etc., and whole cuts such as hams (after removal of skin and bone), picnics, briskets, etc., three times with

Careful mixing with a spatula between each chopping. High fat meat samples, i.e., bacon, pork, sausage, must be nearly frozen before chopping.

C. Samples weighing more than 5 pounds.

1. Chop entire sample using the bowl chopper for approximately 1 minute.

2. Reduce sample by quartering to approximately 2 pounds as follows:

- a. Divide sample in chopping bowl into quarters.

- b. Remove alternate quarters.

- c. Chop for approximately 1 minute.

- d. Repeat quartering until sample is reduced to about 2 pounds, then pass sample through a meat chopper with $\frac{5}{64}$ " plate twice, and mixing between each chopping.

D. Dry Products—chop dry products, such as jerky, using a blender or an homogenizer, after first being coarsely diced, for 15 to 30 seconds to obtain a finely divided sample.

E. Samples for Maximum Internal Temperature—select a sufficiently thick center slice from the center of the canned ham to allow the removal of an 8 to 10 ounce core sample from its center. Chop the entire core sample once; half is to be immediately analyzed for maximum internal temperature and the remaining half retained frozen to be reanalyzed, if necessary, at a later date. If other analyses are required, return the remainder of the center slice to the rest of the ham, including all loose juices, to be prepared as in III B.

F. Split Samples—if emulsified, proceed as in III A. If nonemulsified, no further chopping is required. Mix the sample thoroughly by kneading the sample in its plastic bag just prior to analysis. If a nonemulsified split sample is received that appears to be improperly prepared, the sample must be discarded. The inspector who selected the split sample should be contacted by the Accredited Laboratory Reviewer to resolve sample preparation problems. Split samples are not to be analyzed for maximum internal temperature.

G. Check Samples Received by the Laboratory—unless otherwise specified, mix the check sample, after allowing the temperature to equilibrate, by kneading the sample in its plastic bag. Do not attempt to re chop the sample.

H. Check Samples Originating in the Laboratory—utilize large canned ham samples to prepare a series of identical ham check samples. Chop the canned ham for approximately one minute using a large volume chopper. Pass the entire sample through a meat chopper with $\frac{5}{64}$ " plate twice, with mixing between each chopping. Rapidly bag and seal the completed ham check samples, coding each bag with the same identification and freeze samples immediately. To prepare a cooked sausage check sample series, obtain about 20 pounds of cooked sausage, all one type, i.e., all containing no binders or all containing binders, from the remainders of the sample preparation of regular cooked sausage samples. Proceed in same manner as for ham check samples.

I. Salt Content, Brine Concentration or Water Activity—samples of products labeled: Country Style Ham, Country Ham, Dry Cured Ham, Country Pork Picnic, Country Style Pork Shoulder, Dry Cured Pork Shoulder, or Farm Style Pork Shoulder should be prepared as follows: Select a one-inch center slice and remove all fat and bone, as well as a one-half inch wide strip of meat from the outside of the slice, and discard. The remaining muscle tissue is to be chopped and mixed twice prior to determining the salt content and the brine concentration of water activity.

1.003 Sample Calculation

Converting the results of individual analyses to show added water or added substances involves certain calculations based on the fact that there is a relatively constant ratio between protein and water in the skeletal meat and organs of most animals. The “green weight” of animal tissue is compared to the weight of the finished meat food product by means of this water-protein ratio to determine whether the processor has complied with the requirements for added water or added substances (water plus salt). When the product contains additives, the water-protein ratio becomes more accurately a protein multiplier. In a product in which no added water is permitted, the percent meat protein times the protein multiplier must not be exceeded by the percent total water. The protein multiplier is determined statistically and may vary from 3.6 to 5.8 for different meat products; see sample classification, sections 1.004 and 1.021. The percent meat protein is the percent total protein less an amount equal to the percentage amount of the nitrogen bearing compound times its meat protein equivalent (such as NFDM 0.35, soy flour 0.50, gelatin 1.00 soy protein concentrate 0.70, HVP 0.50, HPP 0.50, MGS 0.50, isolated soya protein 0.90, etc.) present in the product. If the amount of gelatin added to canned product is unknown, 0.6 percent is deducted from the total protein content to obtain the meat protein content. If the amount of MSG, HVP or HPP added to any product is unknown, 0.2 percent is deducted for each one present.

$$\text{Percent Added water} = \text{TW} - \text{NP}$$

$$\text{Percent Added Substances} = \text{TW} + \text{S} - \text{NP}$$

where TW = Percent Total Water

N = Appropriate Protein Multiplier

P = Percent Meat Protein = protein minus the protein due to added non meat nitrogen containing substances.

S = Percent Salt

In order to provide the best estimate for regulatory purposes, all laboratories analyzing cooked sausage official samples are to calculate the added water content by subtracting the meat protein equivalent for all non-meat proteins, except for:

1. Mustard up to 1 percent usage. All use of mustard over 1 percent of the formulation will be subtracted at the rate of 35 percent meat protein equivalent, by multiplying the percentage used minus one percent by 0.35.
2. Autolyzed yeast
3. Cereals and spices

For *all* other non-meat protein additives, the policy stated in Section 1.003 of the USDA “Chemistry Laboratory Guidebook” is in effect. For non-meat proteins not stated in that Section (with the exception of 1, 2, and 3 stated above), subtractions are to be made at the same meat protein equivalent rates as stated in section 1.023 for products regulated under PFF.

1.004 Pork Classification

Interim - Subject to revision

	Protein Multiplier	Added Substances If Appropriate	Added Water If Appropriate
Ham, ham roll: cured, smoked, cooked, dried, spiced (bone-in or boneless)	3.79	0.0	10.0
Ham: cured	3.79	10.0	—
Ham: deviled	3.83	—	0.0
Ham: chopped	3.83	—	3.0
Ham, ham roll, and loins: canned	3.83	8.0	—
Picnics, butts, loins, shoulders, bacon, misc:			
cured	4.00	10.0	—
cured and smoked	4.00	0.0	10
cured and dried	4.00	0.0	10
cured and cooked	4.00	0.0	10
Picnic: canned	3.93	8.0	—
Hams, loins, picnics: sliced	4.00	0.0	—
Water or steamed cooked hams, loin picnics, butts, misc.	4.00	0.0	10.0
Cured pork roll: canned	3.83	8.0	—
Cured pork roll	3.79	0.0	10.0
Sausage:			
Fresh finished	4.00	—	0.0
Fresh finished, water declared	4.00	—	3.0
Franks, weiners, and bologna	4.00	—	10.0
liver, Polish, miscellaneous, and Viennas smoked or cooked, canned			
Luncheon Meat			
No hearts or tongues	3.80	—	3.0
0.1 to 20 percent hearts and tongues	4.00	—	3.0
20.1 to 40.0 percent hearts and tongues	4.20	—	3.0
40.1 to 60 percent hearts and tongues	4.40	—	3.0

Note: See Appendix VI for interim water protein ratios.

1.005 Deviled Ham (MPI Regulations §319.760)

This product is permitted by the Regulations to have a fat content of 35 percent of the finished product and a moisture content equal to that of the fresh unprocessed meat (zero added water). Determine total fat by extraction. Determine moisture and protein by the normal procedures, and use the ratio 3.83, as shown, to calculate added water.

1.006 Perishable Canned Products (MPI Manual, § 18.46)

Certain perishable products are required to have built-in safety factors to protect the consumer. These are the addition of a minimum amount of salt, sodium nitrite, and minimum cooking temperatures. The requirements for canned cured meat loaves, non-specific loaves, and similar products containing no extenders are as follows:

- (a) Cooking temperature 150° F (meat) and 155° F (poultry).
- (b) Brine content 3.5 percent.
- (c) Nitrite ¼ oz (156 ppm) per 100 lb of product.

Canned, cured meat loaves, nonspecific loaves, and similar products containing extenders, must meet the conditions specified above except the brine content must be a minimum of 6.0 percent.

The brine content is calculated by dividing the salt found by analysis by the sum of the salt and water in product. The temperature is specified to destroy trichinae and to destroy the vegetative organisms in the product. When indicated, the cooking temperature may be estimated by the coagulation or phosphatase test.

Imported products in general are required to meet the same standards as their domestic counterparts. Some tests not usually made on domestic products are necessary, because such products are not made under the supervision of a U.S. inspector, and because the history of their preparation is not known.

In addition to the usual request for added substances in imported hams, there is an occasional request to check the "Cooking Temperature." The thermal death point of trichinae is about 137° F. More often, the test is made because the Animal and Plant Health Inspection Service requires a minimum 156° F, which is necessary to destroy any exotic diseases such as food-and-mouth virus present and to prevent entry of that disease into this country. The phosphatase test or the coagulation test, as described in the Part 3.000 may be used to determine the maximum internal temperature, if this request has been made.

Net weight of a canned product is usually considered to be the gross weight less the weight of the can. However, it must be remembered that the net weight of a product packed in a non-nutritive medium, such as brine, does not include the weight of the packing medium. Where cut-out of juice and solids is requested, weigh the solid piece and determine weight of juice by difference.

Report, as an example: Net weight, 4.5 kilograms (10 lb).
Solids, 4.0 kilograms (8 lb, 13 oz).
Juice 0.5 kilogram (1 lb, 2 oz).

Sliced ham, even though derived from canned ham, may not contain added substances since at retail this item is labeled "cooked ham" and is on the same basis as water cooked ham. A water-protein ratio of 4:1 is used to determine added substances. No deduction in protein is to be made for gelatin since the gelatin is scraped off before slicing.

1.007 Fresh Pork Sausage (MPI Regulations; § 319.141)

This item is allowed by the Regulations to be made from trimmings containing up to 50 percent trimmable fat, plus 3 percent seasoning and 3 percent water, when water is declared on the label. Thus 100 parts sausage may contain 0.50 X (100-3-3) or 47 parts fat and 100-3-3-47 or 47 parts water + lean tissue. Of the latter, ⅓ is water and ⅔, or 9.4 parts are meat protein (water-protein ration 4:1). The trimmable fat content is calculated from the protein analysis as follows:

$$(a) \text{ Percent Trimmable Fat } \frac{[100 - ((5 \times \text{percent meat Protein}) + 6)]100}{100 - 6}$$

If water is not declared (a) becomes:

$$(b) \text{ Percent Trimmable Fat } \frac{[100 - ((5 \times \text{percent meat Protein}) + 3)]100}{100 - 3}$$

Attempts are sometimes made to enhance the fresh appearance of pork sausage through addition of sulfite, nitrite, niacin, or ascorbate. Frequent checks should be made for the presence of these substances.

1.008 Canned Vienna Sausage or Frankfurter

Analyses of domestic Vienna sausage or frankfurters packed in barbecue sauce or brine to determine compliance with the 10 percent added moisture limitation must be made on links prior to packing in the can. This should be done by the usual methods employing the 4:1 water-protein.

This requirement does not hold for imported sausages or frankfurters packed in brine because we do not have access to the product prior to packing. The drained weight of the meat product (i.e. franks after brine is drained away) is determined, the declared net weight is noted and the following formula is used to determine the added moisture:

$$M = \frac{\left[\left(\frac{D \times M}{100} - (D - L) \right) \right] 100}{L}$$

$$P = \frac{D \times p}{L}$$

$$AW = M - 4P$$

M = Calculated moisture in product.

P = Calculated protein.

D = Drained weight.

m = Percent moisture by analysis.

L = Labeled weight.

p = Percent total protein by analysis.

AW = added water at time of canning.

It is also required that weight of the sausage in either broth or barbecue sauce be 80 percent of the contents. Products containing less than 80 percent sausage would not be in compliance with requirements.

1.009 Canned Luncheon Meats (MPI Regulations, § 319.260)

These are permitted to contain byproducts whose water-protein ratios are somewhat different from normal meat cuts. Therefore, it is necessary to apply different factors, depending upon the percentage of meat byproducts used. Sections 1.004 and 1.011 show the water-protein ratio to be used for calculating the amount of added water in domestic and import canned pork luncheon meat and pork and beef luncheon meat in 6-pound containers based on the percentage of hearts and tongues. The ratio to be used for calculating the amount of water in luncheon meat in 12-ounce cans is 3.6:1 for beef luncheon meat and 3.8:1 for pork or pork and beef luncheon meat because this size can may not contain hearts or tongues.

1.010 Sausages (MPI Regulations, § 319.180)

When declared on the label, sausages may contain not more than 3.5 percent, individually or collectively, of cereal, vegetable starch, starchy vegetable flour, soy flour, soy protein concentrate, nonfat dry milk, calcium reduced dried skim milk, or dried milk, or 2.0 percent isolated soy protein. In determining the maximum amount of the ingredients that may be used collectively in a product, 2.0 percent of isolated soy protein shall be considered the equivalent of 3.5 percent of any other ingredient. Cooked sausage such as frankfurter, Vienna, and bologna may contain no more than 10 percent added water and no more than 30 percent fat.

1.011 Other Meat Classification

Interim—subject to revision

	<i>Protein Multiplier</i>	<i>Added Substance Limit</i>	<i>Added Water Limit</i>
Hamburger, chopped beef, fresh beef products	3.8	—	0.0
Cooked miscellaneous			
Cooked, smoked beef products	4.0	—	0.0
Pastrami, cooked	3.8	0.0	—
uncooked	4.0	10.0	—
Cured: corned beef brisket	4.0	20.0	—
corned beef round, corned beef, miscellaneous	4.0	10.0	—
Corned beef hash: canned	(See 1.015)		
Beef tongues: cured	4.4	10.0	—
cooked, smoked and/or dried	4.4	0.0	—
Beef Luncheon Meat:			
No hearts and tongues	3.6	—	3.0
0.1–20 percent hearts and tongues	3.8	—	3.0
20.1–40 percent hearts and tongues	4.1	—	3.0
40.1–60 percent hearts and tongues	4.3	—	3.0
Potted Meat Food Products:			
No tripe	4.6	—	0.0
0.1–25 percent tripe	5.0	—	0.0
25.1–50 percent tripe	5.4	—	0.0
50.1–75 percent tripe	5.8	—	0.0
Veal: cuts, ground veal	4.0	—	0.0
Sausage:			
Fresh finished	4.0	—	0.0
Fresh finished, water declared	4.0	—	3.0
Smoked or cooked, canned, franks and weiners, bologna, liver, Polish, miscellaneous and canned Viennas	4.0	—	10.0

Note: See Appendix VI for interim water protein ratios.

1.012 Hamburger (MPI Regulation 319.15(b))

It consists of chopped fresh beef with or without the addition of beef fat and/or of seasoning. It shall not contain any added moisture and not more than 30 percent fat.

Chopped Beef (MPI Regulations; 319.15(a))

This item may contain seasoning, but no added fat or added moisture. Its fat content is also restricted to no more than 30 percent.

1.013 Beef, Tenderized (MPI Regulations; 318.7(c)(4))

Solutions containing approved enzymes and seasoning material may be used for tenderizing beef cuts which are intended for freezing or cooking in the plant. The increase in subweights as a result of this treatment shall not be in excess of 3.0 percent above the weight of the untreated product.

1.014 Cooked Meat (MPI Manual; § 18.35 - .36)

This may be used as a substitute for fresh meat in certain meat products where minimum meat content is based on fresh meat. Establishments preparing cooked meat for use in products may wish to add to the meat product the cooked meat plus the water in which it is cooked and the cooked-out juices. In this case, the weight of the fresh meat would be used by inspectors as a basis to determine the minimum requirements. In case the establishment wishes to use the cooking medium (such as the cooked-out juice) in preparation of some other item, the cooked meat solids alone cannot be considered equivalent to the fresh meats because part of the nutrients in the form of soluble protein and fat are being used elsewhere. Thus, it is necessary to develop a factor relationship between cooked meat solids and fresh meat.

The cooked meat equivalent calculation thus is based on the limiting nutrient, protein. Since a sample of the meat before cooking is usually not available, we assume for calculation purposes that fresh meat contains approximately 18 percent protein, 69 percent water, 12 percent fat, and 1 percent ash. This is based on the 3.8:1 water-protein ratio. Then, if 100 pounds of the fresh meat contains 18 pounds of protein, the equivalent amount of cooked meat solids is that quantity which contains 18 pounds of protein. Thus the quantity in pounds of cooked meat equivalent to 100 pounds of fresh meat is calculated by the equation:

$$\text{Pounds Cooked Meat Equivalent to Pounds Fresh Meat} = \frac{18 \times 100}{\% \text{ Protein}}$$

1.015 Corned Beef Hash (MPI Regulations, § 319.303)

Corn beef hash standards of identity allow not more than 72 percent moisture and 15 percent fat. For calculation purposes we assume that potatoes constitute 50 percent of the product. Because potatoes contain approximately 1.6 percent protein, we deduct 0.8 percent from the total protein found in order to calculate the meat component.

There are two categories of corned beef hash depending upon whether the product was prepared from cooked meat or fresh meat.

(a) Cooked beef must constitute 35 percent or more of the corned beef hash. Meat that was cooked to approximately 30 percent shrink was found to have a water-protein ratio of 2.25 to 1. This 2.25 parts water plus 1 part protein equals 3.25, the cooked meat factor. Because cooked meat consists primarily of meat protein (total protein less potato protein in hash), water, and fat, it is calculated as follows:

$$\text{Percent Cooked Meat} = 3.25 (\% \text{ Protein} - 0.8) + \% \text{ Fat}$$

(b) Fresh meat, or fresh meat with less than 20 percent cooked meat (inspector must note any such mixture on laboratory form), must comprise 50 percent or more of the constituents used to prepare corned beef hash. Fresh beef analysis gives an average of 3.8 parts water, 1 part protein, thus the meat factor is 4.8. Fresh meat content is calculated as follows:

$$\text{Percent Fresh Meat} = 4.8 (\% \text{ Protein} - 0.8) + \% \text{ Fat}$$

1.016 Breaded Cuts (MPI Regulations, § 319.880)

Breading is permitted on beef, pork and/or veal cuts up to 30 percent. The amount may be determined in the laboratory by scraping off the breader and comparing its weight with the total weight. Added water is determined on the meat portion alone because nitrogenous matter and water in the breading would interfere. The inspector normally checks these items; he can determine the quantity of breading material added by weighing the steaks before and after application of the breading material.

1.017 Dinners

Meat components of prepared dinners do not require separation of meat from cereal portion. The whole tamale, enchilada, or breaded item should be considered the meat component and reported as such.

1.018 Pepper and/or Mushroom Steaks

These may be analyzed for added water provided an appropriate deduction is made for water and protein carried into the product by the vegetables. This deduction is based on the inspector's statement on the Form of the amount of peppers or mushrooms present in the product. (A sample without this information may be analyzed but the report should not be made until the information is obtained from the inspector). Peppers and mushrooms each contain approximately 90 percent water and 2 percent protein so these fractions of the vegetables present should be deducted from the total water and total protein respectively before applying the 3.8:1 water-protein ratio to determine added water.

1.019 Potted Meat Food Products (MPI Regulations, 319.761)

Potted meat food products have variable water-protein ratio depending upon the amount of tripe used, as shown in table of Section 1.011. If the amount of tripe used is not known, use the ratio 4.6:1 and so indicate on the report.

1.020 Pickled Pigs Feet (MPI Manual, § 18.48)

This item is required to contain enough acetic acid to maintain pH 4.5 or below in order to be considered a shelf stable item.

1.021 Fats and Oils (MPI Regulations, § 319.702-703)

The analyses normally requested on "Specification" samples of fats and oils are free fatty acids peroxide value, moisture, melting point, smoke point, and anti-oxidants. The free fatty acids and peroxide values are determined by AOCS procedures and are used as criteria for the quality of product. A fat with high free fatty acid may indicate improper handling of fat or ingredients prior to rendering. The peroxide value indicates the degree of rancidity. The active oxygen method (analytical) introduced by King, Roscher and Erwin (Oil and Soap 10 105, 1933) is the official method adopted by the AOCS for estimation of fat stability. This method derived its name from the fact that the criterion of the development of oxidation is the formation of compounds which contain active oxygen and which are reduced by potassium iodide. This method involves heating a sample at an elevated temperature during which time it is aerated with washed air. Heating and aeration are continued until the peroxide content, as determined by the modification of the Wheeler method, reaches certain predetermined values. These values coincide approximately with the point at which the fats are organoleptically undesirable. For vegetable fat, this is considered to be approximately 100 milliequivalents of oxygen per 1000 grams of fat. For lard and beef fat, it is 20 milliequivalents per 1000 grams.

For additional details on application of this procedure, review "Analysis of Fats and Oils" by Mehlenbacher. The antioxidants permitted are those identified in Part 318.7 (c)(4) of the Regulations.

1.022 Cheeseburgers

The following analyses and calculations are required for cheeseburgers:

1. Total Fat—Analyze by Official Methods of Analysis of the AOAC, 13th Edition, 16.255, with the following change to facilitate sample manipulation: Substitute a Mojonnier flask for the small, tall-form beaker.

2. Added water = [Total water-F (meat and cheese protein)]

3. Meat and cheese protein = total protein-(Non-meat + Non-cheese protein). Examples of non-meat protein and non-cheese protein are NFDM, soy derivatives, MSG, etc.

4. The F factor is calculated as follows:

The inspector will state on FSQS Form 6200-1, the weight of meat and cheese in the formulation, and the type of cheese such as:

Meat = 400 pounds

Cheddar Cheese = 80 pounds

The percentage of meat, exclusive of ingredients other than the meat and cheese = $\frac{400}{480} \times 100 = 83.3\%$

The percentage of cheese, exclusive of ingredients other than the meat and cheese = $\frac{80}{480} \times 100 = 16.7\%$

Using Agriculture Handbook No. 8-1, Composition of Foods, find the water and protein contents of the cheese. The name of the cheese must be stated on the form by the inspector. If not, assume the cheese to be cheddar. As an example, if cheddar cheese was used in the above formulation, then Handbook No. 8-1, Item No. 01-009 yields the following data:

water content = 36.75%

protein content (N \times 6.38) = 24.9%

Because the nitrogen to protein factor 6.38 is used and the total protein as analyzed in the product was calculated using 6.25, the cheese protein must be converted by multiplying by 0.98 i.e., $\frac{6.25}{6.38}$.

$$24.9\% \times 0.98 = 24.4\%$$

The moisture protein ratio of cheddar cheese = $\frac{36.75}{24.4} = 1.51 : 1$

That portion of the F factor due to meat = $(0.833)(4) = 3.33$

That portion of the F factor due to cheese = $(0.167)(1.51) = 0.25$

Therefore, the F factor = $3.33 + 0.25 = 3.58$

1.023 Protein Fat-Free (MPI Regulations, 319.19; 319.104; 319.105)

Protein Fat Free (PFF) is a figure derived from laboratory analyses for protein and fat. The following formula is used to determine the PFF of cured pork products from the laboratory findings that are received:

$$\text{PFF} = \frac{\text{Percent Meat Protein by Analysis}}{100 - \text{Percent Fat by Analysis}} \times 100$$

Where:

Meat Protein = Total Protein by Analysis - % Protein due to additive.

Added Ingredients:			<u>% Protein</u>
Soy products	—	Isolated Soy Protein (ISP)	90%
	—	Soy Flour (SF)	50%
	—	Soy Protein Concentrate (SPC)	70%
Fish products	—	Fish Protein Isolate	100%
	—	Fish Protein Concentrate	80%
Yeast products	—	Hydrolyzed yeast	55%
	—	Autolyzed yeast	80%
	—	Yeast Extract	80%
Animal products	—	Whey	15%
	—	Nonfat Dry Milk (NFDM)	35%
	—	Milk Protein Hydrolyzate	91%
	—	Sodium Caseinate	50%
	—	Casein	100%
	—	Bone Protein	100%
	—	Blood Protein	100%
	—	Pork Skin Product	100%
	—	Gelatin	100%
	—	Hydrolyzed Animal Protein	100%
	—	Ham Flavor	100%
	—	Meat Extract	100%
	—	Dried Meat Stocks	100%
Plant products	—	Mustard	35%
	—	Spice mixtures (not to include mustard)	10%
	—	Cereal	10%
	—	Wheat gluten	50%
	—	Hydrolyzed Plant Protein (HPP)	50%
	—	Hydrolyzed Vegetable Protein (HVP)	50%
	—	Mono Sodium Glutamate (MSG)	50%
	—	Vegetable Starch	1%
	—	Starchy vegetable flour	10%

PART 2—NONMEAT PRODUCTS

Non-meat products consist of seasonings, cures, binders, preservatives, etc., that are added to the meat food product, as well as materials coming in contact with the product during processing or packaging. Preparation of these samples for analyses seldom presents problems because most of these items are finely divided solids, liquid solutions, or homogeneous solids such as casings and wrappers. Calculations for most of the determinations are given with the procedure, but a more detailed discussion is in order for certain curing compounds in which it is customary to show the amounts permitted in pickle. Background information on many of the non-meat products is provided to give the analyst a better understanding of the materials with which he works.

2.001 Binders

2.001A. Cereal and Vegetable Flours

Wheat, corn, rye, oats, barley, and milo-maize flours are allowed in appropriate meat products under label declaration of cereal. If a more specific declaration is desired, the individual flour may be identified by name in the ingredient statement of product label. Container label is not required to identify each cereal flour present. Soy, potato, and tapioca flours are also allowed in some products but are not classed as cereal flours; therefore, they must be specifically identified both on container and product label. The term "flour" without further qualification refers to wheat flour. Cereal flours, if improperly handled, are some times found to contain weevils or other insects. Flour should be checked for insects or other contamination by microscopic examination. Gross contamination may be detected by screening flours through a 100-mesh sieve. When necessary, microscopic examination utilizing polarized light may be used to identify specific flours present. Determinations of cereals in meat products may be made by procedures noted in Part 3.000 in this Guidebook.

2.001B. Milk Derivatives

Nonfat dry milk (NFDM) proposed for use in products should be checked to determine whether it is free of extraneous materials and is in compliance with the limits shown. These limits should be used as a basis for acceptance or rejection of samples.

	<u>Percent As Is</u>	<u>Percent Moisture Free</u>
Protein	33.8 - 38.6	35.1 - 40.1
Lactose	43.8 - 55.9	45.5 - 58.0
Ash	6.39 - 9.47	6.63 - 9.83
Fat	0.1 - 1.8	0.1 - 1.9
CaO	1.60 - 2.04	1.66 - 2.12
Water	Less than 5.0 percent	

Titrateable acidity (as lactic acid)—1.3 - 2.0 percent.

Alkalinity of Ash—less than 200 ml 0.1N HCl/100 g.

Scorched Particles—less than 32.5 mg/25 g spray dried.

Scorched Particles—less than 32.5 mg/17 g roller dried.

Not all samples need to be examined for each of the above. Protein and ash analyses should be made on all samples to be sure the sample is nonfat dry milk. Titratable acidity of ash should be determined as quality checks.

The percent “as is” range is given for use without a moisture analysis. However, if analyses fall out of this range, a moisture value should be obtained and the results calculated on a moisture free basis. Calcium-reduced dry skim milk may be used on the same basis as NFDM. Except for the scorched particles and calcium oxide, the above tolerances also apply to this item. Its CaO content should be in the order of 0.6 percent. This material is expected to have a yellow or light tan color and a slightly scorched or toasted flavor. The advantages claimed for this material are greater solubility and less dusting. Mixtures of NFDM or calcium-reduced dry skim milk with other materials are not allowed except in batter and gravy mixes, and breaders.

Container labels of these substances may show the trade names; however, the true identity of the substances must also be shown. Dried whey within the following ranges is permitted in nonspecific loaves, stews, soups and imitation sausage.

Protein	10.5 - 13.0 percent
Lactose	69.0 - 74.0 percent
Calcium	0.75 - 0.90 percent

Whey may not be mixed with other substances prior to acceptance in the plant, except when present as a minor component of batter and gravy mixes and breaders.

Sodium caseinate (90 percent protein) is acceptable for use in nonspecific loaves, soups, and stews. It is not permitted in sausage. It may be mixed with other substances for use in batter and gravy mixes, breaders, and nonspecific items.

2.001C. Soy Preparations

Soy flour, soy protein concentrate, and isolated soy protein are allowed in some meat products. The level of use of the flour and concentrate individually or in combination, is restricted to a maximum of 3.5 percent in sausage. Sausage may also contain a maximum of 2.0 percent isolated soy protein, which is considered to be equivalent to a 3.5 percent level of the other binders. When it is desired to combine isolated soy protein with other binders, calculate the maximum permitted percentage of one by substituting the given percentage of the other in the appropriate equation:

$$B = 3.5 - (I \times 1.75) = \text{lb binder per 100 lb sausage.}$$

$$I = 0.57 (3.5 - B) = \text{lb isolated soy protein per 100 lb sausage.}$$

Soy flour is expected to contain a minimum of 49.5 percent protein, soy protein concentrate a minimum of 69.5 percent, and isolated soy protein a minimum of 89.5 percent, based on the dry weights of the materials. To obtain dry weights, a sample is dried for 2 hours at $130^{\circ}\text{C} \pm 3^{\circ}\text{C}$ in a mechanical convection oven.

To provide a method of control, the isolated soy protein must be tagged with 0.1 percent titanium as titanium dioxide. Procedures for checking these in meat products are given in Part 3.000, and Part 4.000.

2.002 Breeding Mixtures

Breeding mixtures are sometimes found to contain undeclared coloring materials such as dyes, spice oils or extractives, to give the mix the appearance of having been made from corn meal, egg yolk, or butter. This is objectionable because it gives the product a deceptive appearance. Preparation of this nature may contain dyes, spice oils or extractives provided that their presence is declared on the label of the container.

Breeding mixtures may contain cereal, starch, nonfat dry milk, soy derivations with salt, and other usual seasoning materials. The mixture may also contain food-type leavening agents which need only be identified on the label as “leavening agent.”

2.003 Casings

There are two types of collagen casings using in the packing industry; the smaller, which is normally shirred, is used on product such as pork sausage, frankfurters, and some dry sausage, and is intended to be consumed with the product. The other type is used to enclose the larger sausage and is removed before sausage is consumed. Formaldehyde, when used in the manufacture of collagen casings, is usually present both in a free and combined state. As the casings are flushed or soaked prior to use, much of the free formaldehyde is removed. The free formaldehyde is limited to no detectable amount in the edible casing, 10 ppm in inedible casing, and 50 ppm in inedible casing for ring bologna. Combined formaldehyde (total minus free) is limited to 4 ppm in the edible casing and 200 ppm in inedible casing.

Artificial casings intended to impart color to the surface of product must contain only color additives for food use subject to certification by the Food and Drug Administration. It is the inspector's responsibility to determine that the dyes are certified and do not penetrate the product. Colorants present in other artificial casings and wrappers which are not intended to color the surface of the product are not limited to Food, Drugs and Cosmetic (FD&C) dyes but they must be known to be safe in the amounts likely to be found in the product.

Natural casings are shipped to official establishments packed in water solutions of salt, glycerol, or propylene glycol. The latter two solutions are limited to 10 percent concentration. These casings must be rinsed free of packing solution prior to use. Although sulfite and nitrite have not been permitted for treatment of casings, the packing media for preflushed casings is sometimes found to contain a few ppm nitrite which may be present as an impurity in the salt.

2.004 Nettings

Netting material consisting of untreated cotton threads is normally permitted for enclosing products. Cotton strings are also permitted for tying products. Dyes used in strings or netting should not transfer to product and should be suitable for food contact. Some nettings approved for enclosing hams, fresh pork roll, and similar products consist of an elastic innercord covered by cotton thread. These were accepted on the basis of composition data obtained from their manufacturers and upon presentation of evidence showing that the netting did not disintegrate under the usual conditions of use. For products intended to be cooked at home, nettings permitted are those which will withstand the temperatures normally used in cooking or roasting. Nettings are not accepted for use on meat cuts to be exposed to open flame or charcoal fires because they introduce off flavor and leave charred particles on product.

2.005 Paper

Paper used for packing meat products must not deteriorate or contaminate the product. Adhesives, printing inks, and coatings must not migrate from the paper into the product. Inks and dyes used in or on paper or plastic wrappers may not contain cadmium, arsenic, antimony, lead, chromium, mercury, or other harmful substances. Sulfite used in the manufacture of paper and box board has been known to migrate into meat product, thus causing the product to be adulterated. Check such containers from time to time for the level of sulfite.

2.005A. Patty Paper

Paper used to separate hamburger, beef, or pork patties contains formaldehyde. Formaldehyde is used in the production of patty paper and appreciable amounts may be found in the finished paper. The maximum amount of free formaldehyde permitted in the paper is 200 ppm. Because the patty paper is not pre-rinsed or pre-soaked before use, there is no limitation for combined formaldehyde.

2.005B. Giblet Wrap (MPI Regulations 381.66 (d)(11))

The giblet from processed poultry is usually wrapped in paper or bags and inserted into the body cavity. The excess moisture absorbed by the paper will add weight to the carcass. To prevent economic fraud, (1) the amount of moisture absorbed shall be such that the wet weight of the paper shall not exceed 90 pounds per ream, and (2) the amount of moisture absorbed shall not exceed 200 percent of the dry weight of the paper.

2.006 Plastics

The suitability of plastic films or forms for use as wrapping or packaging material may be questioned by an inspector. Unreacted monomers or harmless plasticizers normally present in plastics often give rise to undesirable odors. A decision as to whether or not the odor is objectionable is left to the inspector. Dyes or pigments in or on the plastic must be oil and water-fast to prevent transfer to product. Substances applied to packaging materials which would color the product deceptively or extend its shelf life are not allowed. Small quantities of dextrose, talc, or starch dusted on the plastic to prevent sticking are considered harmless. Cellophane coated with nitrocellulose is acceptable because it permits passage of oxygen from the air.

If the film or foam cannot be identified as one previously approved, it should be forwarded to: Compounds and Packaging Section, Products Safety Branch, FIAD, USDA, FSIS, Science, Building 306, BARC-East, Beltsville, MD 20705.

2.007 Adhesives

The adhesives permitted in connection with packing of product in official establishment normally fall into three categories:

1. Those used in direct contact with product.
2. Those used where contact is not intended and will occur only at the glue line.
3. Those used in a manner which makes contact unlikely.

All adhesives must be composed of edible materials. They must be cleared through the Compounds and Packaging Section, Products Safety Branch, FIAD, USDA, FSIS, Science, Building 306, BARC-East, Beltsville, MD 20705, if there are questions as to their suitability.

2.008 Acceptance of Packaging Materials

The inspector should be informed of the proper procedure required for a supplier or manufacturer to have his packaging or coating material accepted. This concerns materials that form part of a can, carton, casing, or container, that are used as coatings, lubricants, or structural surfaces; or that otherwise come in contact with meat or poultry food products produced under Federal inspection. These materials must be accepted for that use by the Food Ingredient Assessment Division, Science, FSIS, USDA. As in the case of approved labels, packaging components used by, or offered for use by federally inspected establishments should be accompanied by evidence of this acceptance. Thus, the inspector can determine whether or not the packaging material being used presents a hazard to the consumer.

The acceptability of packaging materials is determined by reference to the Food Additives Regulations published by the Food and Drug Administration, U.S. Department of Health and Human Services. These regulations list chemical names of packaging components such as adhesives, coatings, colorants, films, resins, paper, paperboard, etc. A supplier or manufacturer desiring to obtain acceptance of his package component should address his request to:

Compounds and Packaging Section
Products Safety Branch
Food Ingredient Assessment Division
Science, FSIS
U.S. Department of Agriculture
Building 306, BARC-East
Beltsville, MD 20705

He should furnish the following information:

- (a) The brand name or code of the component.

(b) A description of how it will be used.

(c) A list of its ingredients.

The list must identify all major and minor constituents by proper chemical name. Dyes and pigments should be identified by Colour Index Number. Trade secrets and proprietary information will be held in confidence.

When a package component is found acceptable for the proposed use, a letter of acceptance will be issued, referring to the component by brand name or code. The supplier or manufacturer should make this letter available to buyers and inspectors upon request.

Additional information and assistance may be obtained by contacting the Food Ingredient Assessment Division.

2.009 Cleaners, Sanitizers, Pesticides, Boiler Treatment Compounds, etc.

The requirements for acceptance and use of compounds of this nature are prescribed in the "Guidelines for Obtaining Authorization for Compounds to be used in Meat and Poultry Plants." ***List of Proprietary Substances and Nonfood Compounds Authorized for Use Under USDA Inspection and Grading Programs*** lists preparations grouped as follows:

- General cleaning agents
- Cleaners for steam or mechanical devices
- Acid cleaners
- Floor and wall cleaners
- Scouring cleaners
- Sanitizers and sanitizing cleaners
- Water conditioners
- Shell-egg cleaners, destainers, and sanitizers
- Wetting agents for poultry scald vats
- Boiler treatment compounds
- Steam line and cooling system compounds
- Sewage and drain compounds
- Toilet and dressing room compounds
- Liquid hand soaps and hand creams
- Offal room and plant exterior compounds
- Pesticides for various areas and uses
- Fruit and vegetable washing compounds
- Hog scald media
- Tripe denuding agents
- Decharacterizing inks
- Lubricants
- Laundry and Handwashing Compounds
- Paints

Any compound, agent, or preparation specifically appearing in the list may be considered to be approved for use in accordance with applicable meat or poultry inspection requirements. If a sample is received at a field laboratory and it does not appear in the list, it should be forwarded to:

Compounds and Packaging Section
Product Safety Branch
Food Ingredient Assessment Division
Science, FSIS, USDA
Bldg. 306, BARC-East
Beltsville, Maryland 20705

2.010 Curing Agents

2.010A. Nitrite and Nitrate

Sodium nitrite in a product produces the characteristic pink color commonly associated with cured meats. The nitrite itself combines with the red pigment, myoglobin, to form relatively stable pink compound which carries through the smoking and cooking operation. Nitrate is added primarily to provide a reservoir of nitrite. The conversion of nitrate to nitrite is brought about by certain bacteria normally present in meat.

Nitrite is recognized as a potentially harmful substance and, therefore, must be closely regulated. When consumed in significant amount, nitrite reacts with the hemoglobin in the blood and thus prevents it from carrying oxygen to the body. The reaction produces methemoglobin, and published information indicates that if 80 percent of the hemoglobin is reacted, the effects may be fatal. The system of the average adult contains about 700 grams of hemoglobin; therefore, if 80 percent of this or 560 grams is reacted, it may be fatal. It is estimated that 0.6 gram of nitrite is considered sufficient to kill an adult. As the volume of blood in a child is much less, 0.2 to 0.3 grams of nitrite could be dangerous for a child.

Cured meat is allowed to contain 200 ppm of nitrite; plus, if the maximum quantity is present, product will contain 0.09 grams of nitrite in one pound. If this quantity of nitrite in cured meat is doubled or tripled, we begin to approach the dangerous levels of sodium nitrite. Any product found to contain in excess of 200 ppm of nitrite may be harmful, and the laboratory should lose no time in conveying the results of analysis to the inspector for any action he deems advisable. It should be recognized, of course, that accurate results on the determination of nitrite can be obtained only if the sample is properly handled prior to analysis. Heat may cause excessive conversion of nitrate to nitrite or breakdown of nitrite, thereby giving a false picture of the actual nitrite content of product.

Sodium nitrite and potassium nitrite used in a product are limited to ¼-ounce in 100 pounds of chopped meat and/or meat byproduct. One ounce may be used for each 100 pounds of meat in dry salt, dry cure, or box cure. With a 10 percent pump or less, 2 pounds may be used in 100 gallons of pickle. The Regulations specify the amount of nitrate should not be in excess of 2.75 ounces per 100 pounds of chopped meat and/or meat byproduct; not more than 3.5 ounces per 100 pounds of meat in dry cure; and not more than 7 pounds per 100 gallons of pickle.

The formulators of curing compounds are required to state the nitrite content on the container label. Cures declaring nitrite content above 2.0 percent are acceptable if analysis shows actual content not to vary more than 10 percent from figure marked on label. A 15 percent deviation is acceptable for those cures declaring a nitrite content of 2.0 percent or less. If it is a unit cure with a declared nitrite content of 0.5 percent or less, this restriction does not apply provided that the actual amount does not exceed the label claim, and the directions for use are based upon the declared amount.

The following method is used for calculating the maximum amount of cure which may be used, based on the percent of nitrite or nitrate found by analysis:

(1) If the nitrate content is not more than 3.5 times that of nitrite, 200 divided by the percentage of nitrite will give the amount in pounds which may be used per 100 gallons of pickle. If the nitrate is in excess of 3.5 times the nitrite, 700 divided by the percentage of nitrate will give the amount in pounds permitted in (100 gallons) of pickle.

(2) Use same calculations as for pickle above but take one-half of the amount and report as ounces the quantity which may be used for each 100 pounds of meat in dry cure.

(3) To compute the amount of cure in ounces which may be used in 100 pounds of comminuted meat, if the percentage of nitrites is not more than 11 times the nitrite, divide 25.0 by the percentage of nitrite in the cure. If the nitrate is in excess of 11 times the nitrite, then 275 divided by the percentage of nitrate will give the amount of the mixture in ounces which may be added to 100 pounds of comminuted meat or meat byproduct.

Nitrates are not allowed to be mixed with seasonings commercially. They may be added together at the time of processing only. Such mixtures may not be stored in-house.

2.010B. Ascorbic Acid and/or Sodium Ascorbate

Ascorbic acid erythorbic acid may be used in preparation of cooked, cured comminuted meat food products in an amount not to exceed $\frac{3}{4}$ ounce per 100 pounds of meat or meat byproduct. Sodium ascorbate or sodium erythorbate may be used at a level not exceeding $\frac{7}{8}$ ounce per 100 pounds of meat or meat byproduct. A solution containing not more than 10 percent of one of these singularly or in combination may be applied to outer surface of sliced or unsliced cured pork or beef products or comminuted meat food products prior to packaging. Based on a 10-percent or less pump, curing solutions for pork or beef products may contain up to 75 ounces of ascorbic acid or erythorbic acid or 87.5 ounces of sodium ascorbate or sodium erythorbate per 100 gallons of pickle. Sodium citrate or citric acid may replace an amount not to exceed 50 percent of the ascorbic acid, sodium ascorbate, erythorbic acid, or sodium erythorbate.

Proprietary mixtures of seasoning for cured product or curing materials may also contain ascorbic acid, erythorbic acid, or their sodium salts; however, the label for such preparation should show percentage of these materials or contain a statement to the effect that when a given quantity of the preparation is used, the permissible level of the specific material will not be exceeded.

In calculating the quantity of the material which may be added to 100 gallons of pickle for product to be pumped to a level greater than 10 percent, the following formulas may be used:

$$Q = \frac{10A}{P}$$

Where:

Q = quantity in ounces.

A = maximum level of use of acid or salt at 10 percent level of pump.

P = percent pump anticipated.

For example, if it is desired to use maximum amount of ascorbic acid and pump to a 15 percent level, the calculation would be:

$$Q = \frac{10 \times 75 \text{ oz.}}{15} = 50 \text{ oz.}$$

It is impractical to determine the amount which may have been used in product on the basis of recovery from product because of the destruction of these materials during the processing operations. It is desirable, therefore, to attempt to control the material going into product.

2.011 Phosphates

Food grade phosphates have been used in the curing of hams for canning, smoked hams, smoked pork shoulder picnics and canned chopped ham since the early 1950's. Their use in bacon was allowed more recently. Phosphates were accepted for use in these meat food products only after the safety of the chemicals was established. Evidence was also presented to show that phosphates reduce the shrink in product when it is cooked, serve to stabilize the normal cured meat color and have an effect on the texture of the product making it easier to slice. It is recognized that the phosphates improve the moisture holding ability of the meat. Thus, by adjusting the type and amount of phosphate, more added moisture as well as more natural moisture may be retained in product during the processing and cooking operation. The finish product is more juicy, and evidence in MPI indicates that such product is preferred by many consumers. Because the Meat Inspection Regulations limit the amount of added moisture in these particular products, phosphates may not be used to incorporate more water than permitted by the regulations.

Our regulations allow up to 0.5 percent of food grade phosphates in cured pork products. When pumped at a level of 10 percent or less, pumping pickle shall contain not more than 5 percent phosphate. The percentage of each phosphate present in a phosphate mixture is not required to be shown on the container label; however, the identity of each phosphate in the mixture must be shown.

The method contained in this Guidebook should be used in determining phosphate in product. The factor 0.0106 times the percentage meat protein is used to calculate the normal phosphate in meat.

If the type of phosphate used in product is known, the percentage of phosphorus is to be multiplied by the appropriate factor to obtain the figure for that phosphate. *If no information has been furnished about the type of phosphate used, the factor 3.96 for sodium tripolyphosphate should be employed and added phosphate reported accordingly.* The appropriate factor for calculating the anhydrous phosphate for each type of phosphate is listed below:

(1) Disodium phosphate	— 4.58	(7) Dipotassium phosphate	— 5.61
(2) Sodium pyrophosphate	— 4.29	(8) Potassium pyrophosphate	— 5.32
(3) Sodium acid pyrophosphate	— 3.58	(9) Potassium acid pyrophosphate (not allowed)	
(4) Sodium hexametaphosphate	— 3.29	(10) Potassium hexametaphosphate (not allowed)	
(5) Sodium tripolyphosphate	— 3.96	(11) Potassium tripolyphosphate	— 4.82
(6) Monosodium phosphate	— 3.87	(12) Monopotassium phosphate	— 4.39

Because of analytical variation and the variation in natural phosphate content of product, and the possibility that the calculations will not be on the basis of the phosphate actually used, added phosphate findings between 0.5 and 0.59 percent, inclusive, should be reported as 0.5 percent. Findings above 0.59 percent should be reported to the nearest 0.1 percent.

The maximum permissible level of pump with pickle containing 5 percent phosphate is 10 percent. If the product is to be pumped at a level in excess of 10 percent, an appropriate adjustment would have to be made for the amount of phosphate in pickle. For example, if the product is to be pumped at a level of 15 percent of green weight, then the quantity of phosphate which can be used in 100 gallons of pickle can be found by the equation assuming 10 pounds/gallon of pickle):

$$X = \frac{500}{15} = 33.3 \text{ pounds}$$

If some other level of pump is desired, the amount of phosphate to be used in pickle can be determined by substituting the percentage of pump for the figure 15 in the equation.

2.012 Preservatives

Preservatives will be permitted in meat and poultry products only if they are clearly needed. They must not be used in lieu of accepted good manufacturing practices such as adequate sanitation and refrigeration. They must, of course, be accepted food additives, and may not be used to promote deception. Attempts are sometimes made to use ascorbate, niacin, or sulfite to preserve the fresh appearance of the product. These are used to preserve the color of fresh meat rather than for their antibacterial action. This practice is considered deceptive because the consumer is deprived of one of the important criteria used in judging the freshness of product. In addition to these objections, sulfite destroys thiamine (vitamin B₁) present in meats.

Benzoic acid or its sodium or potassium salt is permitted in oleomargarine by the Standards of Identity. It is used to prevent reversion of flavor. Calcium and sodium propionate are permitted in dough used for pizza crust to retard mold growth. Potassium sorbate and propyl para-hydroxy benzoate are allowed on dry sausage to prevent mold. Other preservatives, such as borates and nitrites, may be suspected in fresh products.

2.013 Flavoring and Seasoning Materials

2.013A. Seasonings

A seasoning is a combination of flavoring material or condiments to add zest or variety to foods. The label on a container for seasoning must comply with Federal Regulations. The term "Spices" on the container label may be used to cover a group of spices in a seasoning. The terms "spice oils" and "extractives" may be used to identify these materials in lieu of a more specific declaration. The percentage of monosodium glutamate and hydrolyzed plant protein present in a seasoning must be shown on the container label. When anticaking agents are present in the seasoning, the label must identify the agent. Seasonings consisting wholly or in most part of mustard may not be used in excess of one pound of mustard per 100 pounds of product.

Disodium guanylate disodium inosinate and some of the amino acids are permitted for flavor enhancement of meat products. No specific maximum limit has been set on use of these items, because it is generally felt these are self-limiting.

Liquid paprika concentrate is not allowed in fresh product since this seasoning gives product a deceptive appearance. It makes the product appear to be leaner than actually is the case. Lactose and maltose are not allowed in seasoning, as these interfere with the nonfat dry milk tests in product.

Cereal flours, vegetable flours, nonfat dry milk, and soy derivatives are not allowed in seasoning for products in which the amounts of these are restricted because of the difficulty of identification and control of the substances.

Modified food starch may be used as a carrier for seasonings provided the seasoning is used in such a manner that no more than ½ oz of the starch is added to 100 lb of the meat food. Alpha cellulose may be used as a carrier for seasoning at such a level that the cellulose content of the product does not exceed 2 oz per 100 lb of product. No declaration of either the modified starch or the alpha cellulose is required on label of product. The ingredient statement on the seasoning container label, however, should declare their presence.

Approved vegetable gums may be used in seasonings as stated in 2.019F of this manual. Ascorbic acid is a normal constituent of some spices; up to 1000 ppm is not unusual. This should be taken into consideration when checking spices for added ascorbic acid (see appendix VII).

2.013B. Spices

Acceptable spices are permitted to be used in product either alone or mixed with seasoning materials.

One frequently finds spices adulterated with insect parts, rodent hairs or feces, sand, grit, sheep or goat hairs, or other filth. Economic adulterants include soy grits or flour, beet powder, or spices from which oils have been extracted.

The container label of a mixture of spices and other substances used not identify each spice present as these may be covered under the general term "spices." The compliance, Meat and Poultry standards and labeling Division recognizes the term as being acceptable for such mixture in the ingredient statement on finished product. Spices may be checked for total and acid insoluble ash.

Garlic and onion flakes or powders must be prepared from clean sound plants from which the roots, crowns, and dry outer husks have been removed. This may be determined by microscopic examination. The onion or garlic must be free of filth and insect contamination. Check the onion or garlic for grit or sand by placing one gram of the material into a 40 ml test tube, and adding 15 ml of carbon tetrachloride. If, after the test tube is shaken, no more than three to five small particles of harmless, extraneous matter such as sand settle to the bottom, it is considered acceptable. As a "rule of thumb," onion and garlic of acceptable quality are expected to contain not more than 4.0 percent total ash and 0.2 percent acid insoluble ash.

2.014 Fruits and Vegetables Treated with Sulfite

FSIS policy on the use of sulfite-treated fruits and vegetables is as follows:

Note: Proposed Regulation may change this policy.

(1) Fresh fruits and vegetables may contain before use in a meat or poultry food product, a maximum of 50 ppm sulfite (calculated as SO_2) to prevent darkening.

(2) Dehydrated fruits and vegetables may contain before use in a meat or poultry food product, a maximum of 200 ppm sulfite (calculated as SO_2) to prevent darkening.

(3) Dehydrated fruits and vegetables intended for use in a meat product where the meat component is separated from the non-meat component may contain, before use in a meat or poultry food product, a maximum of 2500 ppm sulfite (calculated as SO_2)

2.015 Sweetening Agents

The following sweetening agents are permitted in appropriate products.

Sugars:

Invert sugar—mixture of equal parts of glucose and fructose.

Fructose—produced by hydrolysis of sucrose.

Corn syrup—mixture of maltose, dextrose, water and dextrins derived from corn.

Sugar—sucrose from juice of sugar cane or sugar beets.

Maple sugar—sucrose with sap flavoring from maple tree.

Honey—glucose and levulose from nectar of flowers.

Corn syrup solids—mixture of maltose, dextrose and dextrins derived from corn.

2.016 Artificial Sweeteners

Saccharin is allowed for sweetening certain products. See Part 318.7(c)(4) of the Regulations.

2.017 Corn Syrup

Corn syrup solids and corn syrup are permitted in comminuted products up to a level of 2 percent based on the weight of the corn syrup solids—2.5 percent syrup is equivalent to 2 percent solids. These items are permitted in pumping pickle at a level not in excess of 50 pounds of solids or 60 pounds of syrup per 100 gallons of pickle.

Because the maltose content used as a basis for laboratory control of these items in product varies from shipment to shipment, it is necessary to obtain a sample of the material used when analyzing product for the amount present. In this manner, the actual maltose factor for the specific lot may be obtained. The analytical procedure for checking this in product is given in Part 3.000.

2.018 Smoke Flavors

Certain smoke and imitation smoke flavors made by processes which eliminate harmful substances are accepted for use in meat products. Before a smoke flavor is accepted, its safety must be demonstrated by the manufacturer. To do this, he must provide:

(1) A detail description of the manufacturing process and data to show it can and will be manufactured in a manner to produce material as represented.

(2) Data to show the absence of certain polyaromatic hydrocarbons and the method used to obtain this data.

Approved smoke flavoring or approved imitation smoke flavoring may be mixed with water, animal fats or vegetable oils, salt, sugar, dextrose, hydrolyzed plant protein, and acceptable seasoning materials prior to entering official establishments. A small amount of polysorbate 80 and/or vinegar may be present to stabilize the solution.

Smoke preparations have been accepted from several manufacturers. These preparations may be used by other firms to formulate seasonings and flavorings. Samples of smoke preparations received at field laboratories which are not equipped to check them for harmful substances should be forwarded to the Food Ingredient Assessment Division for evaluation.

2.019 Miscellaneous Materials

2.019A. Anticaking Agents

As anticaking agents, salt, cures, or seasonings may contain up to 2 percent singly or in combination of tricalcium phosphate, tetrasodium pyrophosphate, calcium carbonate, magnesium carbonate, calcium stearate, silica gel, calcium aluminum silicate, calcium silicate, magnesium silicate, sodium aluminosilicate, sodium calcium aluminosilicate, sorbitol, glycerol (glycerin), silicon dioxide, and propylene glycol.

The ingredient statement on the label of containers of such preparations should identify the agent present. When preparations containing these are used in the product, the presence of anticaking agents need not be shown on product label.

Up to 13 parts per million of yellow prussiate of soda (sodium ferrocyanide decahydrate) is accepted as a crystallizing agent in salt. The container label should show it by statements such as "Yellow Prussiate of Soda Added."

2.019B. Antifoaming Agents

Dimethylpolysiloxane (350 centistokes viscosity) may be added in an amount not to exceed 10 ppm as an antifoaming agent to shortening containing meat fats intended for deep fat frying. Its presence must be declared on the label as follows: "Dimethylpolysiloxane, an Antifoaming Agent. added."

2.019C. Antifrosting Agents

Ethylene glycol and propylene glycol are accepted for defrosting cooling coils of refrigerator systems under certain conditions. Ethylene glycol is permitted providing the temperature of the exhaust air from the coils is not in excess of 10° F. Propylene glycol is accepted over the wide range of temperatures expected in the area. Use of either compound must be regulated so that the concentration in the atmosphere of a room is no more than 2 ppm.

2.019D. Coatings and Paints

Those substances permitted for coating metal containers and drums are identified in the appropriate Food Additives Regulation published by the Food and Drug Administration. The can coatings must be applied and cured in a manner so as to result in a smooth coating having the physical properties to serve the intended use. The coatings for lard drums must be

applied to a clean, sandblasted or otherwise specially prepared surface and baked at a temperature of approximately 400° F so as to result in a smooth, hard and odorless coating. Containers and drums are checked by the inspector before use and those found to be unsatisfactorily coated are rejected. Compounds of chromium, cadmium, arsenic, lead or other metals known to form toxic salts are not permitted in coating formulations. Questions which the field laboratories do not feel qualified to answer should be referred to the Washington office. Any request for use of materials in coating formulations, other than those identified in the Food Additives Regulations, should be referred to the Washington office for consideration.

Paints permitted in plants on walls, ceilings, floors, and that portion of equipment not directly contacting product are required to be formulated from compounds known to be safe as described in *Accepted Meat and Poultry Equipment* publication. Compounds of cadmium, chromium, arsenic, antimony, barium (except barium sulfate), and other compounds known to be toxic are not allowed in paint formulations. Lead is prohibited as a pigment or drier. Some paints are permitted to contain certain fungicidal substances, but mercury compounds are not permitted. (See 2.009)

Certain resinous coating materials have been accepted on contact surfaces of equipment when determined by the Washington office to be chemically acceptable for this purpose. Inquiries concerning paints or coatings should be forwarded to the Washington office (see 2,008), unless the laboratory has ample evidence that preparations have previously been approved or consist of those materials that are known to be safe. While the Food Ingredient Assessment Division determines the chemical suitability of a paint, the inspector in the plant has the authority and responsibility to determine whether or not a specific paint is appropriate for application to a surface or area.

2.019E. Enzymes

The enzymes permitted for tenderization of product are papain, ficin, bromelin, the protease from *Aspergillus oryzae* and the *Aspergillus-oryzae-flavus* group. Up to two percent glycerin or propylene glycol may be used to stabilize the solution. Acetic acid, citric acid, lactic acid or sodium bicarbonate may be used to adjust the pH. Hydrolyzed plant protein or monosodium glutamate may be present as flavoring. The label covering such preparation must identify all ingredients.

Samples of tenderizers submitted with requests for determination as to tenderizing effects should be referred to:

Microbiologist
Branch Chief
USDA-FSIS-Science
Food Microbiology Branch
Bldg. 318, ARC-East
Beltsville, MD 20705

2.019F. Gums

The vegetable gums—acacia, locust bean, guar, and tragacanth—are permitted for emulsifying and thickening gravies, salad dressings, sauces, seasonings, breaders, and batter mixes. Carrageenan, the gelatinous extract of seaweed called carrageen or Irish Moss, is also allowed. Chemically it is a complex carbohydrate made up of galactose, dextrose, levulose, and small quantities of pentosan. Seasoning preparations containing these gums for use in sausage are restricted to a level that will not result in more than 0.15 percent gum in product. The vegetable gums are all similar in composition and use. The identity of these substances must be shown on the container label.

2.019G. Lubricants

Food-grade animal or vegetable fats, refined mineral oil, and petrolatum are allowed on processing equipment where direct contact with product occurs. Only refined mineral oils having no taste, odor, or appreciable amount of fluorescence are allowed for application to contact surfaces of processing machinery to prevent rust after cleaning or as lubricant where some contact is expected. Aluminum, calcium, sodium greases, or other less refined oils may be used where no significant contact is expected. Aluminum, calcium sodium greases, or other less refined oils may be used where no significant contact is expected. Lubricants in this category may also contain graphite, bentonite, or other harmless substances. Compounds of lithium may not be used where contact with food product may be expected. When the laboratory receives a sample of lubricant for which no information on composition or prior official approval is available, it should be forwarded to the Compounds Evaluation Unit for evaluation. (See Section 2.009 for address.)

2.019H Sodium Bicarbonate-Sodium Carbonate

Sodium bicarbonate is permitted in dry cure mixtures containing nitrite with ascorbic acid, erythorbic acid, or their sodium salts. The amount present should not exceed 3 ounces of bicarbonate per $\frac{1}{4}$ ounce of nitrite, or $\frac{7}{8}$ ounce of the sodium salts, or $\frac{3}{4}$ ounce of the acids.

Sodium carbonate may be used in cures on the same basis as sodium bicarbonate except the amount is $1\frac{1}{2}$ ounces per $\frac{1}{4}$ ounce of nitrite, or $\frac{7}{8}$ ounce of the sodium salts, or $\frac{3}{4}$ ounces of the acids.

Sodium bicarbonate may also be used in treatment of water used for pumping pickle to condition the water and to remove certain undesirable salts. The alkalinity of the resulting pickle may not be in excess of pH 8. It is also permitted in certain acid type meat foods to adjust the acidity, in the rendering of fats, and for cleaning vegetables.

2.019I. Dried Yeast

Dried yeast (*Saccharomyces cerevisiae*) and dried torula yeast (*Candida utilis*), food grade, have been accepted for use in appropriate product for flavoring purposes. Smoked yeast may also be used in any meat food product in which yeast is an expected ingredient and which has been smoked or contains smoked meat.

2.019J. Sorbital

Sorbitol, a polyhydric alcohol, is accepted for use in those cooked sausages labeled frankfurter, frank, furter, weiner, or knockwurst as an aid to (1) improve flavor, (2) ease the removal of casings from these products, and (3) reduce charring and caramelization during the cooking process.

PART 3—MACROANALYTICAL PROCEDURES

3.001 Moisture Determination

In this determination, a weighed sample is heated, cooled, and then reweighed. The loss in weight is calculated as moisture content.

3.001A. Theory

Although this procedure is quite simple and straightforward, a few precautions are necessary.

- (a) Weigh the sample as rapidly as possible to minimize loss of moisture.
- (b) The weight of the pan should include a paddle which is used in spreading the sample across the bottom of the pan, thereby presenting a greater sample surface area which is beneficial to moisture removal.
- (c) If the sample is relatively dry when received, a small quantity of distilled water may be added to the pan only *after* the sample weight is obtained. This quantity of water will be helpful in spreading the sample across the bottom of the pan, and will introduce no error since it will be evaporated when the sample is oven-dried.
- (d) Do not overload the drying oven or sample may be insufficiently dried and give low results. Use the oven's booster heater, if the oven is so equipped, to minimize this recovery time. Drying time will start when the original temperature has been reached.

3.001B. Apparatus

Covered aluminum dish. At least 50 mm diameter and not greater than 40 mm deep, containing a paddle.

Mechanical convection oven. Preferably one equipped with a booster heater.

3.001C. Determination

Accurately weigh sample (representing approximately 2 g of dry material) into a covered aluminum dish. Dry, with lid removed, for 16 - 18 hours at 100 - 102°C, or for four hours at 125°C, in mechanical convection oven. Cool and weigh.

3.001D. Calculations

$$\text{Percent} = \frac{100(B - C)}{A}$$

A = sample weight

B = weight of dish + sample prior to drying

C = weight of dish + sample after drying

Note: If laboratory is not air-conditioned, and humidity is high, it is advisable to desiccate dishes prior to the initial and final weighings.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

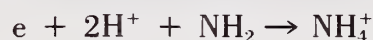
3.002 Protein Determination

Total protein content is determined by the Kjeldahl procedure, in which nitrogen is converted to ammonium bisulfate during digestion, and ammonia liberated therefrom by sodium hydroxide. The ammonia is collected in an excess of standard acid solution, and the excess acid titrated with standard alkali solution.

3.002A. Theory

Approximately 2 g of meat, concentrated sulfuric acid, HgO and K₂SO₄ are added to the Kjeldahl flask. When this mixture is boiled, the protein hydrolyzes to gelatin and finally to amino acids, the basic structural units of proteins and the end result when the protein is completely hydrolyzed.

A solution of NH₄HSO₄, HgO and KHSO₄ in concentrated sulfuric acid results from the digestion. An essential amino acid, threonine, has the formula CH₃ · CHOH · CHNH₂ · COOH. In order to convert the NH₂ group to NH₄⁺, reducing conditions must exist, as shown by the following partial equation:



The hot acid causes charring and the carbon thus formed is the reducing agent which acts upon the amino groups, converting or reducing them to NH₄HSO₄.

The HgO is a catalyst, speeding up the reaction considerably. K₂SO₄ is added to raise the boiling point.

Following cooling and dilution of the digest NaOH, Na₂S₂O₃ and zinc or pumice are added and the NH₃ is distilled into a receiver containing an excess of standard acid. The flask should not be shaken until connected to the distillation assembly to prevent loss of NH₃.

The NaOH will release NH₃ from NH₄HSO₄ by double displacement, and the Na₂S is employed to precipitate HgS (black, brown, orange or yellow depending upon the NaOH concentration). If it were not precipitated, the Hg⁺⁺ would tie up the NH₃ as a Hg amido complex, and low results would be obtained.

Zinc acts as an anti-bumping agent by slowly reacting with the NaOH and forming nascent hydrogen. It is this fine stream of hydrogen that prevents super-heating, the main cause of bumping. Use of Zn, however, probably results in distillation of metallic Hg (nascent H is an excellent reductant). To avoid this, commercial preparations containing HgO, K₂SO₄, and pumice are available and may be used.

The distillate must be on the acid side of the indicator, which may be methyl red, methyl purple, or bromphenol blue. The excess acid is then titrated with standard NaOH and the protein calculated as follows:

$$\text{Percent Protein} = \frac{(V_a - V_b)(1.4007)(6.25)(N)}{\text{Sample Weight}}$$

Where V_a = Volume of standard acid added

V_b = Volume of standard base used in titration

1.4007 = Meq. wt. of nitrogen (includes factor of 100 for percentage)

6.25 = Factor for converting from nitrogen to protein because protein is 16 percent nitrogen

N = Normality of standard acid and base

If both the acid and base are 0.2000N, the formula reduces to:

$$\text{Percent Protein} = \frac{(V_a - V_b)(1.75)}{\text{Sample Weight}}$$

The last equation indicates the importance of being sure that the normalities of the standard solutions are as close to 0.2000N as is practical; 0.1996 to 0.2004 is acceptable. The factor 1.75 assumes that standard solutions of 0.2000N strength are being used.

If the 1.75 factor is used to calculate protein content, and the standard solutions are appreciably higher than 0.2000N, the calculated results will be lower than the true protein content of the sample. Conversely, if the standard solutions are appreciably lower than 0.2000N, and the 1.75 factor is used, the calculated results will be higher than the true protein content of the sample.

3.002B. Apparatus

- (a) Kjeldahl digestion: distillation equipment
- (b) Erlenmeyer flask: 500 ml
- (c) Kjeldahl flask: 800 ml
- (d) Filter paper: 7 cm Whatman #541 or equivalent

3.002C. Reagents

(a) Kjeldahl catalyst: 15 g K_2SO_4 + 0.7 g HgO (Commercially prepared catalysts are available containing pumice, if desired)

(b) Sulfuric Acid: A.C.S.

(c) NaOH Solution: prepare 1200 ml of (1 + 1) NaOH. Allow to stand until clear (ca 10 days)

(d) Metallic Zinc: Powder, A.C.S., to be used if catalyst does not contain pumice

(e) Indicator solution: Fleisher Methyl Purple or equivalent

(f) Acid potassium phthalate: N.B.S. Standard

(g) Standard NaOH Solution: $0.2000 \pm 0.0004N$

Add 108 ml of (1 + 1) NaOH to CO_2 -free distilled H_2O and dilute to 10 liters. Standardize against potassium acid phthalate, using Phenolphthalein indicator.

(h) Standard acid solution: $0.2000 \pm 0.0004N$. Prepare either hydrochloric or sulfuric acid solution:

(i) Hydrochloric Acid: Dilute 178 ml of 35 - 37 percent reagent grade HCl to 10 liters. Standardize against standard NaOH solution, and adjust strength accordingly.

(j) Sulfuric Acid: Dilute 55 ml of 98 percent reagent grade sulfuric acid to 10 liters. Standardize against standard NaOH solution, and adjust strength accordingly.

(k) Sodium Hydroxide: Sodium Thiosulfate solution.

Dissolve 460 g of $Na_2S_2O_3 \cdot 5H_2O$ in water; dilute to one liter with water, and add this solution to 15,250 g of NaOH dissolved in 14,250 ml of water. This will yield 20 liters of a 50 percent (w/w) NaOH solution. If other volumes are desired, adjust weights of NaOH and $Na_2S_2O_3 \cdot 5H_2O$ accordingly. The specific gravity of the final solution should be at least 1.45.

3.002D. Determination

- (a) Accurately weigh, by difference, ca 2 grams of sample on a circle of nitrogen free filter paper, fold and transfer to an 800 ml Kjeldahl flask.
- (b) Add catalyst and 40 ml of concentrated H_2SO_4 .
- (c) Digest on Kjeldahl apparatus until solution is clear, and then for at least 30 minutes longer. When digestion is complete, volume of acid solution in flask should just cover the area of heating element exposed by the porcelain refractory.
- (d) Allow flask and contents to cool to room temperature; carefully add 400 ml of cold tap water and mix by swirling. Again allow flask and contents to cool to room temperature. If catalyst did not contain pumice, add a pinch of zinc powder.
- (e) To a 500 ml Erlenmeyer flask, add 25.0 ml of 0.2000N HCl or H_2SO_4 , methyl purple indicator, and sufficient distilled H_2O so the end of the delivery tube is submerged. Place the Erlenmeyer flask in position and turn on the heater and the condenser water.
- (f) Add 90 ml of $\text{NaOH}-\text{Na}_2\text{S}_2\text{O}_3$ solution down the side of the Kjeldahl flask, so that it layers on the bottom. Connect the flask to the distilling bulb on the condenser carefully; swirl flask to mix contents thoroughly and place flask on hot heater.
- (g) Collect 150-200 ml of distillate, and titrate the excess standard acid with 0.2000N NaOH .

3.002E. Calculations

$$\text{Total Protein Content: Percent} = \frac{(A - B)(1.75)}{C}$$

A = Volume of Standard 0.2000N acid added.

B = Volume of Standard 0.2000N NaOH used in titration.

C = Sample Weight.

1.75 = Factor derived as shown under THEORY.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

3.003. Alternate Protein Determination

The Kjeldahl Method for Protein in Meat Using Boric Acid Traps

This method involves heating the material for some time with concentrated sulfuric acid at or near its boiling point in the presence of a catalyst followed by a distillation in alkaline solution, catching the nitrogen converted to ammonia in a boric acid trap and titrating it directly with standard acid.

3.003A. Theory

See 3.002A.

3.003B. Apparatus

- (a) Kjeldahl digestion: distillation equipment
- (b) Erlenmeyer flask: 500 ml
- (c) Kjeldahl flask: 800 ml
- (d) Filter paper: 7 cm Whatman #541 or equivalent

3.003C. Reagents

- (a) Kjeldahl catalyst: 15 g K_2SO_4 + 0.7 g HgO (Commercially prepared catalysts are available containing pumice, if desired)
- (b) Sulfuric Acid: A.C.S.
- (c) NaOH Solution: prepare 1200 ml of (1.1) NaOH. Allow to stand until clear (ca 10 days)
- (d) Metallic Zinc: Power, A.C.S., to be used if catalyst does not contain pumice
- (e) Mixed indicator - Mix 2 parts of 0.05% methyl red (in 95% ethanol with 3 parts 0.075% bromocresol green (in 95% ethanol). or N-Point Indicator (MCB #NX847)
- (f) Acid potassium phthalate: N.B.S. Standard
- (g) Standard NaOH Solution: $0.2000 \pm 0.0004N$

Add 10.1 ml of (1 + 1) NaOH to CO_2 -free distilled H_2O and dilute to 1 litre. Standardize against potassium acid phthalate, using Phenolphthalein indicator.

- (h) Standard acid solution: $0.2000 \pm 0.0004N$. Prepare either hydrochloric or sulfuric acid solution.
- (i) Hydrochloric Acid: Dilute 178 ml of 35-37 percent reagent grade HCl to 10 liters. Standardize against standard NaOH solution, and adjust strength accordingly.
- (j) Sulfuric Acid: Dilute 55 ml of 98 percent reagent grade sulfuric acid to 10 liters. Standardize against standard NaOH solution, and adjust strength accordingly.
- (k) Sodium Hydroxide: Sodium Thiosulfate solution.

Dissolve 460 g of $Na_2S_2O_3 \cdot 5H_2O$ in water; dilute to one liter with water, and add this solution to 15,250 g of NaOH dissolved in 14,250 ml of water. This will yield 20 liters of a 50 percent (w/w) NaOH solution. If other volumes are desired, adjust weights of NaOH and $Na_2S_2O_3 \cdot 5H_2O$ accordingly. The specific gravity of the final solution should be at least 1.45.

(l) Saturated boric acid soln - 60 g H_3BO_3 /liter - Stir well and let sit over night before use. Filter before use and add 20 ml mixed indicator per liter of solution (e).

3.003D. Determination

(a) Accurately weigh by difference, ca 2 grams of sample on a circle of nitrogen free filter paper, fold and transfer to an 800 ml Kjeldahl flask. (Run a blank of 1 g sucrose along with and the same way as samples.)

(b) Add catalyst and 40 ml of concentrated H_2SO_4 .

(c) Digest on Kjeldahl apparatus until solution is clear, and then for at least 30 minutes longer. When digestion is complete, volume of acid solution in flask should just cover the area of heating element exposed by the porcelain refractory.

(d) Allow flask and contents to cool to room temperature; carefully add 400 ml of cold tap water and mix by swirling. Again allow flask and contents to cool at room temperature. If catalyst did not contain pumice, add a pinch of zinc powder.

(e) To a 500 ml Erlenmeyer flask, add 50.0 ml of saturated boric acid-mixed indicator solution, and sufficient distilled H_2O so the end of the delivery tube is submerged. Place the Erlenmeyer flask in position and turn on the heater and the condenser water.

(f) Add 90 ml of $\text{NaOH-Na}_2\text{S}_2\text{O}_3$ solution down the side of the Kjeldahl flask, so that it layers on the bottom. Connect the flask to the distilling bulb on the condenser carefully; swirl flask to mix contents thoroughly and place flask on hot heater.

(g) Collect 150-200 ml of distillate, and titrate the blank first, with 0.2000N HCl or H_2SO_4 , to a gray-pink end point. Match the sample end point with the blank end point color.

3.003E. Calculations

$$\text{Total Protein Content: Percent} = \frac{(A - B) (1.75)}{C}$$

A = Volume of Standard 0.2000N acid used in sample titration

B = Volume of Standard 0.2000N acid used in blank titration

C = Sample Weight

1.75 = Factor derived as shown under THEORY (3.002A.)

References:

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition

Kjeldahl, Z., Anal. Chem. 22: 336-82 (1883)

Wilferth, Chem. Zentr. 56: 17, 113 (1885)

Winkler, Z. Angew. Chem. 26: 231 (1913)

Winkler, Z. Angew. Chem. 27: 630 (1914)

3.004 Automated Determination of Nitrogen

3.004A. Theory

NH_3 reacts with hypochlorite and phenate ion in alkaline solution to produce quinonechloramine which reacts with additional phenate ion, producing the blue dissociated form of indophenol with maximum absorbance at 630 nm in alkaline solution.

3.004B. Apparatus

(a) Automatic analyzer. AutoAnalyzer with following modules (Technicon Instruments Corp.); Sampler II; proportioning pump I; continuous digester; proportioning pump II; current stabilizer; constant temperature bath equipped with variable temperature regulator (set at 70°C); colorimeter with 15 mm tubular flowcell, 630 nm filters, and No. 9 aperture; voltage stabilizer; recorder with transmittance paper; vacuum pump; 2 manifolds (Figures 1 and 2); and 8.5 ml sample cups.

(b) Pipet: Automatic zeroing, 50 ml (Kontes Glass Co., K-763280).

(c) Tubing: Fluran 1'-5000, 0.125" id, Acidflex, or Teflon, 0.133" id.

(d) Pipetting machine: Automatic Model 60453 with Model 70327 valve syringe (BBL, Division of BioQuest).

3.004C. Reagents

(a) Vanadium Pentoxide solution: Weigh 40.0 g NaOH pellets and transfer to 1 liter volumetric flask. Add 500 ml H_2O , dissolve, and cool. Add 12.5 g V_2O_5 to flask, dissolve, dilute to volume, and mix.

(b) Digestion mixture: Caution:—Mix in order 150 ml V_2O_5 solution 90 ml 60-62 percent HClO_4 , and 3460 ml H_2SO_4 . Rate of consumption is 497 ml/hr.

(c) Wash solution - H_2SO_4 (1 + 1). Caution: To 1 liter H_2O in 2 liter volume flask, add 1 liter H_2SO_4 slowly with swirling. Cool to room temperature, dilute to volume with H_2O . Rate of consumption is 234 ml/Hr.

(d) Alkaline tartrate solution: Dissolve 150 g Potassium Sodium tartrate Na tartrate. $4\text{H}_2\text{O}$ in 1950 ml H_2O and add 1050 ml 50 percent NaOH solution. Consumption rate is 174 ml/hr.

(e) Sodium hypochlorite solution: 4 to 6 percent NaOCl (Fisher Scientific Co.). Consumption rate is 25 ml/hr.

(f) Alkaline phenol solution: Prepare 15N NaOH solution by adding 2400 ml 50 percent NaOH (w/w to 600 ml H_2O , cooling, and storing in polyethylene bottle. To 500 ml 15N NaOH in vessel cooled by circulating cold H_2O , slowly add 276 ml 90 percent liquid phenol. Cool to room temperature and dilute to 1 liter. Store in dark in polyethylene bottle. Consumption rate is 48 ml/hr.

(g) Dilution water.

(1) Input manifold: Consumption rate is 468 ml/hr.

(2) Analytical manifold: Consumption rate is 234 ml/hr.

3.004D. Preparation of Standard

Grind freeze-dried beef (available from Wilson Certified Foods, Inc., P.O. Box 7345, Omaha, NE 68107) four times in standard laboratory mill (Straub Co., Croydon, PA 19020), Model 4-E, or equivalent). Store ground material in freezer to prevent deterioration. Determine Kjeldahl nitrogen using 0.3-0.5 g. Based on nitrogen content, prepare standards containing 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, and 4.0 mg N/ml as follows: Into 9 separate 1.5 liter beakers, transfer

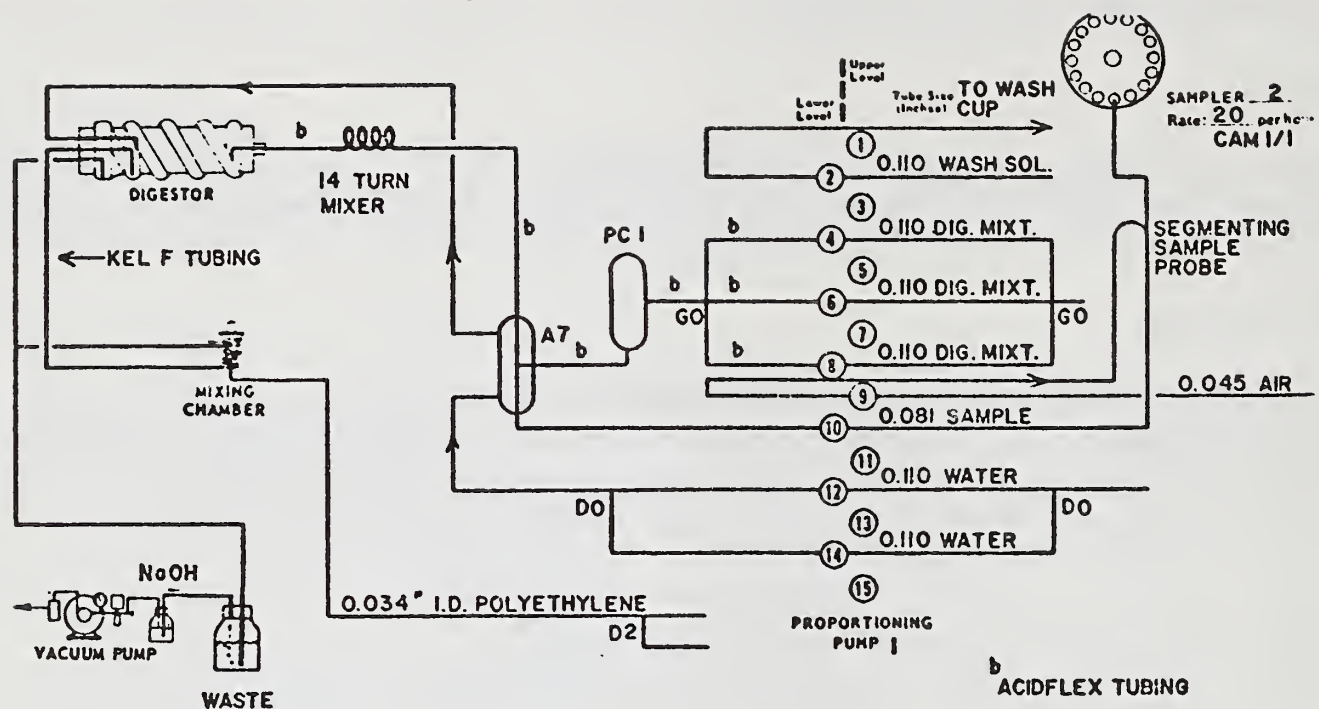


Figure 1.—Helix inlet manifold.

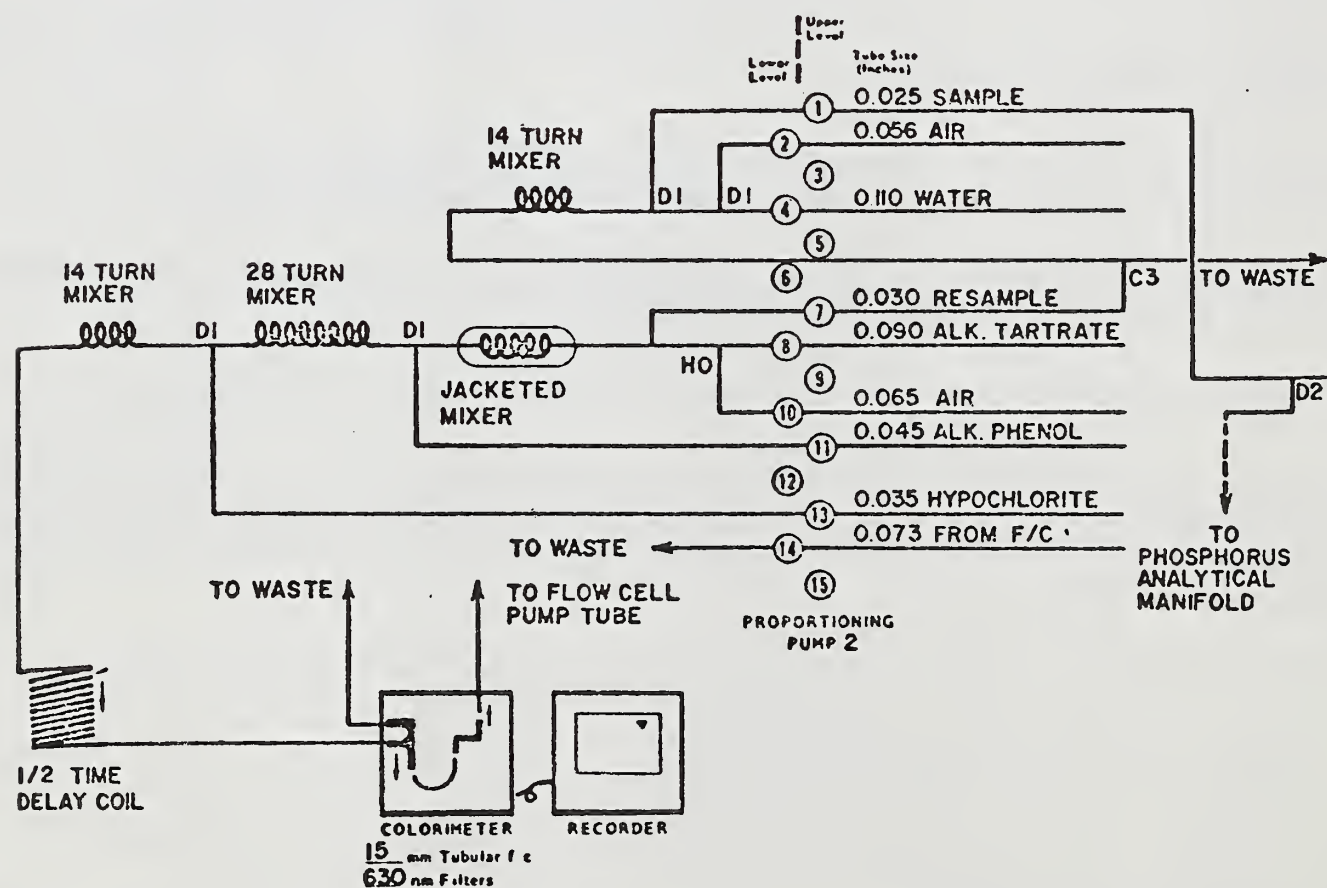


Figure 2.—Nitrogen analytical manifold.

weighed amount freeze dried beef. Add 400 ml H₂O and disperse thoroughly, using magnetic stirrer. Slowly add, with stirring, 500 ml H₂SO₄ and continue stirring 15 minutes. Cool in ice bath until fat solidifies. With aid of stirring rod and funnel, transfer solution through glass wool pad into 1 liter volume flask. Let warm to room temperature, dilute to volume, and mix. Store in polyethylene bottle. Solution is stable \geq 75 days.

3.004E. Preparation of Sample

Accurately weigh 10.00 g sample into 200 ml tallform beaker. Pipet 53 ml H₂O into beaker with pipetting machine. Add 1 inch Teflon-coated stirring bar, cover with 60 mm watch glass, and disperse sample, using magnetic stirrer. With stirring, add 50 ml H₂SO₄ using automatic pipet, and continue stirring until sample is dissolved. Cool to room temperature in cooling bath.

3.004F. Analytical System

Use standard 0.0625 inch id transmission tubing throughout system unless otherwise specified. Pump sample at 2.5 ml/min and segment with air pumped at 0.8 ml/min. Pump digestion mixture at 8.28 ml/min through PCl fitting and add to sample at A7 fitting. Pass sample stream through 14-turn mixing coil into inlet of digester helix. Aspirate diluted sample into bubble chamber and remove aliquot for analysis at rate of 0.23 ml/min. Dilute aliquot with H₂O pumped at 3.90 ml/min and segment with air at 1.20 ml/min. Pass stream through 14 turn mixer and C3 debubbler, and resample at 0.32 ml/min. Add alkaline tartrate solution at 2.90 ml/min and air at 1.60 ml/min. Then pass stream through jacketed mixer, add alkaline phenol solution at 0.80 ml/min, and pass through double mixer. Add NaOCl solution at 0.42 ml/min and pass stream through 14 turn mixer and ½ time-delay coil for color development. Finally pass stream into colorimeter with 630 nm filter and 15 mm tubular flowcell into waste at 2.00 ml/min.

3.004G. Start-Up Procedure

Place all reagent lines, except Acidflex, in water; turn on both proportioning pumps and digester power. Turn on vacuum pump, setting gage at 12-15 psi. Pump digestion mixture and all analytical reagents through their respective lines to determine that system is operating properly. Prior to routine use, optimize digester unit as follows: Using 2.0 mg N/ml standard in duplicate, vary amperage setting according to following table, and record absorbance. Allow 20 minutes interval after changing setting to stabilize helix temperature before standard is analyzed. Use settings giving highest absorbance.

<u>Amperage Settings</u>		<u>Amperage Settings</u>	
Stage 1	Stages 2 and 3	Stage 1	Stages 2 and 3
2.50	3.00	4.20	6.40
3.00	4.00	4.50	7.00
3.50	5.00	5.00	8.00
3.80	5.60	5.50	9.00
4.00	6.00		

Set digester helix to rotate at 6.7 rpm, referring to Technicon Manual T-69-123 (1970) for instructions.

3.004H. Determination

- Pour standard and prepared samples into 8.5 ml cups and place in Sampler II turntable.
- Adjust sampling rate to 20/hr, with 1:1 sample-to-wash ratio to provide 1.5 min sampling and 1.5 min wash.
- Press reset button and activate sampler turntable, thus passing standards and samples into analytical system. Place stop bar in turntable. (Formation of excessive fat deposits in sample line between segmenting sample probe and input manifold can be retarded by passing wash solution through double mixer wrapped with heating tape and covered with layer of aluminum foil and layer of asbestos; adjust temperature to 60° with variable transformer connected to heating tape.)

(d) Read absorbance of samples from recorder chart and compare with standard curve of absorbance against mg N/ml. Include standard curve with every 30 samples.

(e) As 53 ml H₂O + 50 ml H₂SO₄ added to samples gives 95 ml (8 ml contraction), it may be assumed that 10 g samples containing 50% H₂O give final volume of 100 ml.

(f) However, certain dry products (e.g., pepperoni) or wet products (e.g., corned beef brisket) may contain considerably more or less than 50 percent causing an error by as much as 0.6 percent protein. Close approximation may be obtained by adding H₂O content of sample, as determined in §3.001, to 95 ml to obtain final total volume. Using this assumption,

$$\%N = [(\%H_2O \text{ in sample } 10) + 95] \\ \times (\text{mg N/ml}) \times 0.01.$$

$$\% \text{ Protein} = \% N \times 6.25$$

3.004I. Shut-down Procedure

Turn off heat switch and let first stage temperature reach 200°C. Remove helix cover and place all reagent lines except digestion mixture in H₂O after first stage temperature is 150°C. Place digestion mixture line in empty Erlenmeyer and let Acidflex pump tubes "air-wash." Rinse entire system for 15 minutes. Shut off proportioning pumps and break vacuum in liquid waste bottle. Turn off digester power switch and replace helix cover.

3.005 Fat Determination (Ether Extraction)

This method involves a partial drying of a weighed sample prior to a Soxhlet extraction. The extracted fat is weighed and the fat content calculated.

3.005A. Theory

Sample weights are obtained by difference in order to minimize changes in the moisture content, which will adversely affect the fat content. Regardless of which procedure described below is used, it is important that sand be incorporated with the sample before drying. The purpose of the sand is to create a greater surface area, necessary to remove moisture and prevent entrapment of fat.

Excessive drying may oxidize the fat, and give high results.

3.005B. Apparatus

- (a) Thimbles: fat extracted 25 × 80 mm.
- (b) Soxhlet extraction apparatus: I.D. of extraction tube, 30 mm.
- (c) Filter paper: 9 cm Whatman #541 or equivalent.
- (d) Aluminum dishes, disposable: approximately 60 mm. diameter × 18 mm deep.
- (e) Glass beads, hollow, perforated: 4 mm diameter.

3.005C. Reagents

- (a) Petroleum ether: AOCS Specification H 2-41. or AOAC 10.118, 12th Edition.
- (b) Sea sand: washed and ignited.

3.005D. Determination

(a) (1) Accurately weigh, by difference, 3 to 4 grams of sample into a thimble lined with a circle of filter paper and containing a small amount of sand.

(2) Mix sand and sample with a glass rod, wipe glass rod with filter paper strip(s), and place strip(s) in thimble.

(3) Place thimble and contents in a 50 ml beaker and dry in a mechanical convection oven for 6 hours at 100-102°C, or for 1-½ hours at 125°C. Proceed as in (c) below.

(b) (1) Accurately weigh, by difference, 3 to 4 grams of sample into a small disposable aluminum dish.

(2) Add a small amount of sand, and with the aid of a small aluminum or glass paddle, spread the mixture across the bottom of the dish.

(3) Dry as in (a) above, roll edges of dish and insert into a thimble. Proceed as in (c) below.

(c) (1) Accurately weigh an extraction flask containing a few glass beads.

(2) Extract the sample (contained in the thimble from (a) or (b) above) with petroleum ether for 4 hours at a condensation rate of at least 5-6 drops per second, in a Soxhlet extraction apparatus. (If sample has been dried as in (a) above, rinse the 50 ml beaker with three 10-ml portions of petroleum ether and add rinsings to the extraction tube).

(3) At the completion of the extraction, place flask on a steam bath and evaporate the petroleum ether until no odor of it is detectable.

(4) Dry flask and contents in a mechanical convection oven for time required to obtain constant weight at 100°C, cool and weigh.

Note: Laboratory must have data available to support time used to obtain constant weight. If these data are available, analysts may use the specific time, rather than remove, cool, and weigh several times for each sample.

3.005E. Calculations

$$\text{Fat content, percent} = \frac{100(B - C)}{A}$$

A = Sample weight

B = Weight of flask after extraction

C = Weight of flask prior to extraction

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

3.006 Fat Determination by Specific Gravity

3.006A. Theory

This method utilizes the Foss-Let fat analyzer which is based on the principle of a density measurement of a tetrachloroethylene extract of the fat in a magnetic float cell. The sample is first homogeneously prepared in a "Reactor," which breaks down the sample, squeezing out the oil while simultaneously absorbing the moisture in the sample with calcium sulfate.

3.006B. Apparatus

- (a) Foss-Let apparatus and accessories: dispenser, cooler, "Reactor" and measuring unit (Foss America, Inc., P.O. Box 504, Fishkill, NY. 12524.).
- (b) Top Loading Balance—capable of weighing $45\text{g} \pm 0.1\text{g}$ samples.
- (c) Schleicher & Schuell (S&S) No. 589, 7 cm diameter filter paper.

3.006C. Reagents

- (a) Tetrachloroethylene (perchloroethylene), available from local dry cleaning suppliers.
- (b) Calcium Sulfate, anhydrous, or Plaster of Paris (available from local hardware stores).

3.006D. Determination

- (a) Allow Foss-Let potentiometer to obtain maximum stability by turning the instrument on with chamber filled with perchloroethylene at least two hours before operation. *Note:* Perchloroethylene vapors are harmful to inhale and handling of this liquid should be confined to a fume hood.
- (b) Daily calibrate the Foss-Let potentiometer by using perchloroethylene to set the zero point before samples are analyzed. The zero point must read 000 ± 001 ; adjust side knob of instrument using a screw driver. Calibrate the instrument by using Foss-Let mineral oil (specific gravity at 23°C -0.915) to set control for 50% fat point at 850.0 reading. The swimmer must rise at a digital setting of 850 ± 003 . Adjust side knob of instrument using a screw driver. Record zero point and 50% fat point readings in Standards Record Notebook.
- (c) Daily, adjust compensator of perchloroethylene dispenser to correspond with the ambient temperature.
- (d) Weigh a $45.0\text{g} \pm 0.1\text{g}$ sample into a stainless steel extraction-chamber cup.
- (e) Set brass hammer with ring etched bottom on the spindle in the cup. Check to assure the bore of the hammer is less than or equal to 11 millimeters diameter or do not use.
- (f) Add 80g of Plaster of Paris (approximately 3 g) or 60g of anhydrous calcium sulfate to the cup. For very wet samples more may be required to obtain clear filtrate (not milky).
- (g) Dispense perchloroethylene $120\text{ ml} \pm 0.1\text{ ml}$ into the cup using the dispenser unit. Place the cover tightly on the cup.
- (h) Put the extraction cup onto the "Reactor" and shake for two minutes.
- (i) Assemble the filtration device by first placing a 7 cm circle of S&S Number 589 filter paper into perforated base. Place filtration device at top of measuring chamber of Foss-Let analyzer.

(j) Remove the cup from the shaker and lift the cover. Pour the liquid extract through the assembled filtration device into the Foss-Let potentiometer measuring chamber. Filtrate must be clear, not cloudy. Allow the filtration to continue at least until the extract appears in the overflow clear tubing to the waste container and 10 ml of filtrate is retained in the measuring chamber.

(k) Remove the filtration device, slide the viewing lens to the right into position and allow the temperature control lamp to switch off before taking a reading of the specific gravity of the crude fat extract in perchloroethylene. If the temperature in the measuring chamber is too low, the temperature control lamp is on. If the lamp is flashing, the temperature is too high. *Warning:* Do not move the viewing lens to the left since this automatically releases the drain valve.

(l) When the measuring chamber is at the correct temperature, push the black swimmer reset button and view the movements of the hydrometer through the viewing lens. Turn the digital knob clockwise (towards a higher readings) slowly, just until swimmer rises. Repeat reading three times and record the mean digital read-out on a worksheet or in a workbook.

(m) Convert the digital read-out figure to a percent fat reading (2 decimal places) using the conversion table (e.g., read-out of 99 is equivalent to 5.00 percent fat). Round the percent fat reading to the nearest 0.1 percent.

(n) Move the viewing lens to the left and allow the retained filtrate to drain into a plastic 10 liter waste bottle. Rinse the measuring chamber three times with approximately 40 ml of clean perchloroethylene. Depress the red drain valve button so the waste will drain completely into the 10 liter container.

(o) Clean the extraction cup, cup cover, brass hammer and filtration unit thoroughly, assuring proper waste disposal procedures under the fume hood. Allow the washed items to thoroughly dry before re-use.

CONVERSION TABLE

Foss-Let Units

	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
0	0	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
10	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90	0.95
20	1.00	1.05	1.10	1.15	1.20	1.25	1.30	1.35	1.40	1.45
30	1.50	1.55	1.60	1.65	1.70	1.75	1.80	1.85	1.90	1.95
40	2.00	2.05	2.10	2.15	2.20	2.25	2.30	2.35	2.40	2.45
50	2.50	2.55	2.60	2.65	2.70	2.75	2.80	2.85	2.90	2.95
60	3.00	3.05	3.10	3.15	3.20	3.25	3.30	3.35	3.40	3.45
70	3.50	3.55	3.60	3.65	3.70	3.75	3.80	3.85	3.90	3.95
80	4.00	4.05	4.10	4.15	4.20	4.25	4.30	4.35	4.40	4.45
90	4.55	4.60	4.65	4.70	4.75	4.80	4.85	4.90	4.95	5.00
100	5.05	5.10	5.15	5.20	5.25	5.30	5.35	5.40	5.45	5.50
110	5.55	5.60	5.65	5.70	5.75	5.80	5.85	5.90	5.95	6.00
120	6.10	6.15	6.20	6.25	6.30	6.35	6.40	6.45	6.50	6.55
130	6.60	6.65	6.70	6.75	6.80	6.85	6.90	6.95	7.00	7.05
140	7.10	7.15	7.20	7.25	7.30	7.40	7.45	7.50	7.55	7.60
150	7.65	7.70	7.75	7.80	7.85	7.90	7.95	8.00	8.05	8.10
160	8.15	8.20	8.25	8.30	8.35	8.40	8.50	8.55	8.60	8.65
170	8.70	8.75	8.80	8.85	8.90	8.95	9.00	9.05	9.10	9.15
180	9.20	9.25	9.30	9.35	9.40	9.50	9.55	9.60	9.65	9.70
190	9.75	9.80	9.85	9.90	9.95	10.00	10.05	10.10	10.15	10.25
200	10.30	10.35	10.40	10.45	10.50	10.55	10.60	10.65	10.70	10.75
210	10.80	10.85	10.90	10.95	11.05	11.10	11.15	11.20	11.25	11.30
220	11.35	11.44	11.45	11.50	11.55	11.60	11.65	11.75	11.80	11.85
230	11.90	11.95	12.00	12.05	12.10	12.15	12.20	12.25	12.30	12.40
240	12.25	12.50	12.55	12.60	12.65	12.70	12.75	12.80	12.85	12.90

CONVERSION TABLE

Foss-Let Units

	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
250	12.95	13.05	13.10	13.15	13.20	13.25	13.30	13.35	13.40	13.45
260	13.50	13.55	13.65	13.70	13.75	13.80	13.85	13.90	13.95	14.00
270	14.05	14.10	14.15	14.25	14.30	14.35	14.40	14.45	14.50	14.55
280	14.60	14.65	14.70	14.80	14.85	14.90	14.95	15.00	15.05	15.10
290	15.15	15.20	15.30	15.35	15.40	15.45	15.50	15.55	15.60	15.65
300	15.70	15.75	15.85	15.90	15.95	16.00	16.05	16.10	16.15	16.20
310	16.25	16.35	16.40	16.45	16.50	16.55	16.60	16.65	16.70	16.80
320	85	16.90	16.95	17.00	17.05	17.10	17.15	17.20	17.30	17.35
330	17.40	17.45	17.50	17.55	17.60	17.65	17.75	17.80	17.85	17.90
340	17.95	18.00	18.05	18.10	18.20	18.25	18.30	18.35	18.40	18.45
350	18.50	18.60	18.65	18.70	18.75	18.80	18.85	18.90	18.95	19.05
360	19.10	19.15	19.20	19.25	19.30	19.35	19.45	19.50	19.55	19.60
370	19.65	19.70	19.75	19.95	19.90	19.95	20.00	20.05	20.10	20.15
380	20.25	20.30	20.35	20.40	20.45	20.50	20.55	20.65	20.70	20.75
390	20.80	20.85	20.90	20.95	21.05	21.10	21.15	21.20	21.25	21.30
400	21.40	21.45	21.50	21.55	21.60	21.65	21.70	21.80	21.85	21.90
410	21.95	22.00	22.05	22.15	22.20	22.25	22.30	22.35	22.40	22.50
420	22.55	22.60	22.65	22.70	22.75	22.85	22.90	22.95	23.00	23.05
430	23.10	23.20	23.25	23.30	23.35	23.40	23.45	23.55	23.60	23.65
440	23.70	23.75	23.80	23.90	23.95	24.00	24.05	24.10	24.15	24.25
450	24.30	24.35	24.40	24.45	24.55	24.60	24.65	24.70	24.75	24.80
460	24.90	24.95	25.00	25.05	25.10	25.20	25.25	25.30	25.35	25.40
470	25.50	25.55	25.60	25.65	25.70	25.75	25.85	25.90	25.95	26.00
480	26.95	26.15	26.20	26.25	26.30	26.35	26.45	26.50	26.55	26.60
490	26.65	26.75	26.80	26.85	26.90	26.95	27.00	27.10	27.15	27.20

CONVERSION TABLE

Foss-Let Units

	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
500	27.25	27.35	27.40	27.45	27.50	27.55	27.65	27.70	27.75	27.80
510	27.85	27.95	28.00	28.05	28.10	28.15	28.25	28.30	28.35	28.40
520	28.50	28.55	28.60	28.65	28.70	28.80	28.85	28.90	28.95	29.00
530	29.10	29.15	29.20	29.25	29.35	29.40	29.45	29.50	29.55	29.65
540	29.70	29.75	29.80	29.90	29.95	30.00	30.05	30.10	30.20	30.25
550	30.30	30.35	30.45	30.50	30.55	30.60	30.70	30.70	30.80	30.85
560	30.90	31.00	31.05	31.10	31.15	31.25	31.30	31.35	31.40	31.50
570	31.55	31.60	31.65	31.75	31.80	31.85	31.90	32.00	32.05	32.10
580	32.15	32.20	32.30	32.35	32.40	32.45	32.55	32.60	32.65	32.70
590	32.80	32.85	32.90	32.95	33.05	33.10	33.15	33.21	33.30	33.35
600	33.40	33.50	33.55	33.60	33.65	33.75	33.80	33.85	33.90	34.00
610	34.05	34.10	34.15	34.25	34.30	34.35	34.40	34.50	34.55	34.60
620	34.65	34.75	34.80	34.85	34.95	35.00	35.05	35.10	35.20	35.25
630	35.30	35.35	35.45	35.50	35.55	35.65	35.70	35.75	35.80	35.90
640	35.95	36.00	36.05	36.15	36.20	36.25	36.35	36.40	36.45	36.50
650	36.60	36.65	36.70	36.80	36.85	36.90	36.95	37.05	37.10	37.15
660	37.25	37.30	37.35	37.40	37.50	37.55	37.60	37.70	37.75	37.80
670	37.85	37.95	38.00	38.05	38.15	38.20	38.25	38.35	38.40	38.45
680	38.50	38.60	38.65	38.70	38.80	38.85	38.90	39.00	39.05	39.10
690	39.15	39.25	39.30	39.35	39.45	39.50	39.55	39.65	39.70	39.75
700	39.85	39.90	39.95	40.00	40.10	40.15	40.20	40.30	40.35	40.45
710	40.50	40.55	40.60	40.70	40.75	40.80	40.90	40.95	41.00	41.10
720	41.15	41.20	41.30	41.35	41.40	41.45	41.55	41.60	41.65	41.75
730	41.80	41.85	41.95	42.00	42.05	42.15	42.20	42.25	42.35	42.40
740	42.45	42.55	42.60	42.65	42.75	42.80	42.85	42.95	43.00	43.05

CONVERSION TABLE

Foss-Let Units

	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
750	43.15	43.20	43.30	43.35	43.40	43.50	43.55	43.60	43.70	43.75
760	43.80	43.90	43.95	44.00	44.10	44.15	44.20	44.30	44.35	44.40
770	44.50	44.55	44.60	44.70	44.75	44.80	44.90	44.95	45.05	45.10
780	45.15	45.25	45.30	45.35	45.45	45.50	45.55	45.65	45.70	45.80
790	45.85	45.90	46.00	46.05	46.10	46.20	46.25	46.35	46.40	46.45
800	46.55	46.60	46.65	46.75	46.80	46.85	46.95	47.00	47.10	47.15
810	47.20	47.30	47.35	47.45	47.50	47.55	47.65	47.70	47.75	47.85
820	47.90	48.00	48.05	48.10	48.20	48.25	48.35	48.40	48.45	48.55
830	48.60	48.65	48.75	48.80	48.90	48.95	49.00	49.10	49.15	49.25
840	49.30	49.35	49.45	49.50	49.60	49.65	49.70	49.80	49.85	49.95
850	50.00	50.05	50.15	50.20	50.30	50.35	50.40	50.50	50.55	50.65
860	50.70	50.75	50.85	50.90	51.00	51.05	51.15	51.20	51.25	51.35
870	51.40	51.50	51.55	51.60	51.70	51.75	51.85	51.90	52.00	52.05
880	52.10	52.20	52.25	52.35	52.40	52.50	52.55	52.60	52.70	52.75
890	52.85	52.90	53.00	53.05	53.10	53.20	53.25	53.35	53.40	53.50
900	53.55	53.60	53.70	53.75	53.85	53.90	54.00	54.05	54.15	54.20
910	54.25	54.35	54.40	54.50	54.55	54.65	54.70	54.80	54.85	54.90
920	55.00	55.05	55.15	55.20	55.30	55.35	55.45	55.50	55.55	55.65
930	55.70	55.80	55.85	55.95	56.00	56.10	56.15	56.25	56.30	56.40
940	56.45	56.50	56.60	56.65	56.75	56.80	56.90	56.95	57.05	57.10
950	57.20	57.25	57.35	57.40	57.50	57.55	57.65	57.70	57.75	57.85
960	57.90	58.00	58.05	58.15	58.20	58.30	58.35	58.45	58.50	58.60
970	58.65	58.75	58.80	58.90	58.95	59.05	59.10	59.20	59.25	59.35
980	59.40	59.50	59.55	59.65	59.70	59.80	59.85	59.95	60.00	60.10

3.007 Salt Determination

The sodium chloride content is determined by the well-known Volhard method. The sample is treated with AgNO_3 , then wet-ashed, and the excess AgNO_3 is back-titrated with KCNS.

3.007A. Theory

This method embodies many interesting principles of inorganic analytical chemistry. The AgNO_3 solution must be added first, followed by the concentrated HNO_3 . *This order of addition is critical to ensure complete precipitation of the chlorides.* If HNO_3 is added first, loss of chloride by volatilization as HCl could occur because HCl has higher vapor pressure than HCO_3 .

The volume of AgNO_3 solution added must be in excess of that required to react with the chlorides in the sample.

The concentrated solution of KMnO_4 is added to oxidize any organic matter not disposed of by the HNO_3 . Should too much KMnO_4 be accidentally added, the addition of small quantities of sugar will cause color removal.

Following boiling, cooling and dilution, add diethyl ether and back-titrate the excess AgNO_3 with KCNS solution, employing ferric ammonium sulfate solution as an indicator.

After all the silver has been back-titrated, an excess of thiocyanate may react with the precipitated AgCl because the solubility product of AgCNS is $1/100$ that of AgCl .

$$s_{\text{AgCNS}} = 1.0 \times 10^{-12}$$

$$s_{\text{AgCl}} = 1.1 \times 10^{-10}$$

The addition of nitrobenzene or diethyl ether overcomes this difficulty by coating the precipitated AgCl , thereby withdrawing it from the action of the thiocyanate solution.

The $\text{FeNH}_4(\text{SO}_4)_2$ reacts with an excess of thiocyanate, forming the salmon-colored complex, ferric thiocyanate FeCNS^{++} , indicating the end point.

3.007B. Reagents

- (a) Ferric alum indicator - Saturated aqueous solution of reagent grade $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$.
- (b) Silver Nitrate - 0.100N - Dissolve 17.04 g of AgNO_3 , previously dried at 110°C , in distilled water, and dilute to 1 liter. Standardize (using excess AgNO_3) against 0.100N NaCl (5.845 g per liter) according to Volhard or Mohr method.
- (c) Potassium Thiocyanate - 0.100N - Dissolve 9.72 g of reagent grade KCNS in distilled water, and dilute to 1 liter. Verify the strength of this solution as follows: Pipette 25 ml of standard AgNO_3 solution into a 300 ml Erlenmeyer flask, add 80 ml of distilled H_2O , 15 ml of 1 + 1 HNO_3 and 2 ml of the ferric alum indicator. Titrate with KCNS solution to a permanent light brown (salmon-colored) end point. The ratio of the volume of KCNS to the volume of AgNO_3 should be 1:1.
- (d) Potassium Permanganate - 5 percent aqueous solution.
- (e) Diethyl ether - reagent grade.

3.007C. Determination

- (a) Weigh 2.5-3 g of finely comminuted and thoroughly mixed sample into a 300 ml Erlenmeyer flask.

(b) Add 25.0 ml of 0.100N AgNO_3 solution, swirl flask until sample and solution are in intimate contact, and then add 15 ml of conc. HNO_3 .

(c) Add sufficient boiling chips and boil until meat dissolves; add KMnO_4 in small portions; continue boiling under color disappears and solution becomes almost colorless.

(d) Add 25 ml of H_2O , boil for 5 minutes, cool, and dilute to ca 150 ml with H_2O .

(e) Add ca 5 ml of diethyl ether, 2 ml of the ferric alum indicator, and shake vigorously to coagulate the precipitated AgCl .

(f) Titrate the excess AgNO_3 with KCNS solution to a permanent, salmon-colored end point.

3.007D. Calculations

$$\text{Percent NaCl} = \frac{(25.0 \text{ ml} - \text{ml KCNS})(0.1\text{N})(5.85)}{\text{Sample Weight}}$$

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

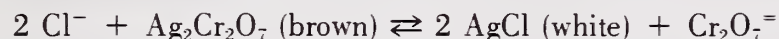
3.008 Indicating Strip Method (Salt)

(Quantab Chloride Titrator)

This procedure is suitable for determining the salt content (as chloride) of meat and meat product.

3.008A. Theory

The chloride in hot aqueous solution reacts with silver dichromate, turning the $\text{Ag}_2\text{Cr}_2\text{O}_7$ from brown to white. Capillary action carries the aqueous solution upward to react with a moisture sensitive indicator to show the completion of the titration.



3.008B. Apparatus

- (a) Quantab chloride titrator — No. 1176 (Ames Co., Div. of Miles Laboratories, Inc.), (range: 0.3% — 10%)
- (b) Beaker — 150 ml
- (c) Graduated cylinder— 100 ml
- (d) Fluted filter paper — 7cm

3.008C. Determination Caution Quantabs should be checked with standard NaCl solution.

- (a) Weigh 10 grams of comminuted product directly into a 150 ml beaker.
- (b) Add 90 ml of hot water (ca 70° C) and stir thoroughly to extract the salt. Cool.
- (c) Place a filter paper, cone down, directly into the extract.
- (d) Place a Quantab into the filtrate above the filter paper.
- (e) When the blue strip appears remove the Quantab.
- (f) Compare reading from Quantab to calibrated table provided to get percent salt. Dilution factor (usually 10) must be used.

NOTE: Some meat products may coagulate, thus preventing complete extraction of the available salt. In those cases, one of the following steps may be followed:

- (1) Weigh 10 grams of sample and add 90 ml of water (room temperature) to the contents of the beaker, slurry, and bring to a boil.
- (2) Weigh 10 grams of sample and add 10 ml of water (room temperature) to the contents of the beaker, slurry, and add 80 ml of hot water and mix.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

3.009 Phosphate Determination

In this procedure, a partially dried sample is ashed, the phosphates then hydrolyzed to the ortho form, and separated as quinolinium phosphomolybdate.

3.009A. Theory

This method seems very similar to the ammonium phosphomolybdate procedure, but is actually quite different.

Phosphomolybdic acid is formed first (in the presence of citrate), which then forms quinolinium phosphomolybdate (QPM) with the base, quinoline. The citrate in the reagent complexes any ammonium ion, thereby preventing the precipitation of ammonium phosphomolybdate.

The original version of this procedure required two separate solutions in order to form the QPM precipitate: a citric-molybdic acid solution and a quinoline solution. The inclusion of acetone permitted these two solutions to be combined, so that a single reagent could be employed as the precipitant. This reagent is known as the quimociac reagent, and derives its name from QUInoline, MOlybdate, Citrate, and ACetone constituents of the mixture.

3.009B. Apparatus

- (a) Gooch crucible: Coors No. 4
- (b) Glass fiber filter paper: 2.4 cm circles

Preparation of the crucible: Place Gooch crucible containing a glass fiber filter disk in suction apparatus. Center dish and wash with approximately 50 ml H_2O . Dry crucible at 250°C for 30 minutes in a forced-draft oven, cool in desiccator and weigh.

3.009C. Reagents

- (a) Dilute Nitric Acid: 1 volume concentrated HNO_3 + 4 volumes H_2O
- (b) Quimociac Reagent: Dissolve 70 grams sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in 150 ml H_2O . Dissolve 60 grams citric acid monohydrate in a mixture of 85 ml of concentrated HNO_3 and 150 ml H_2O and cool. Gradually add the molybdate solution to the citric-nitric acid solution while stirring. Dissolve 5 ml synthetic quinoline, with stirring, in a mixture of 35 ml of concentrated HNO_3 and 100 ml H_2O . Gradually add this solution to the molybdic-nitric acid solution, mix well, and let stand for 24 hours. Filter, add 280 ml acetone, dilute to 1 liter with H_2O , and mix. Store in either a non-colored polyethylene bottle or a dark brown glass bottle.

3.009D. Determination

- (a) Weigh accurately about 2.5 grams (no more than 780 mg QPM) of sample into an ashing dish and dry for 30 minutes at 125°C in a forced-draft oven.
- (b) Ash at 550°C until white ash is obtained.
- (c) Cool, add 25 ml of dilute nitric acid and heat on steam bath for 30 minutes.
- (d) Filter into a 500 ml beaker, wash dish and paper with distilled water so that total volume in the beaker is approximately 100 ml.
- (e) At this point, run a reagent blank in parallel, using 25 ml of dilute nitric acid and 75 ml of distilled H_2O .
- (f) Add 50 ml of quimociac reagent, cover with a watch glass, and boil for one minute (do not use an open flame).

(g) Cool to room temperature while swirling carefully, transfer the precipitate to the prepared crucible, and wash 5 times with 25 ml portions of distilled H₂O), allowing each portion to drain thoroughly (use suction) before adding the next portion.

(h) Dry crucible and contents for 30 minutes at 250°C, cool in desiccator and weigh.

3.009D.1. Wet Ashing (Optional)

The following wet ashing procedure may be used as a screening method. Volative samples must be repeated by the above procedure.

(a) Weigh ca 2.0 g comminuted sample into a 200 ml Kohlrausch or sugar flask, using filter paper to wrap the sample to prevent the meat from adhering to the neck of the flask.

(b) Add 5 ml of the concentrated hydrochloric acid, and 30 ml of concentrated nitric acid to the flask. At this point prepare a reagent blank using a filter paper and the acids.

(c) Place flask on a hot plate (in a hood) and digest the sample until approximately 15 ml of solution remains. *Caution! Do not let go to dryness.*

(d) Cool flask in hood; make to volume with distilled water. Use the bottom of the fat layer as the meniscus. Mix thoroughly.

(e) Filter a portion, ca 30 ml, of the solution through a filter paper and pipette a 25 ml aliquot (.25 g) into a 500 ml beaker. Add 75 ml of distilled H₂O.

(f) Proceed as in step (e) of "Determination."

3.009E. Calculations

$$\text{Phosphorus Content} = \frac{[(100)(A - B)(0.014)]}{C} - 0.0106 (\% \text{ Meat Protein})$$

Where:

A = Weight of sample precipitate

B = Weight of blank precipitate

C = Sample weight

0.014 = Gravimetric factor derived from:

Atomic Weight of Phosphorus = 30.97

Molecular Weight of (QPM) = 2212.71 = (C₉H₇N)₃ H₃PO₄·12MoO₃

$$\frac{P}{\text{QPM}} = 0.014$$

0.0106 = Factor to correct for the natural phosphorus content of meat protein

Phosphate content = (Phosphorus Content) (F)

$$F = \frac{\text{Anhydrous Molecular Weight of desired phosphate}}{(X) (\text{Atomic Weight of Phosphorus})}$$

Where X = number of atoms of phosphorus in one molecule of the phosphate

The following table lists phosphates and their corresponding factors.

<u>Sodium Phosphates</u>	<u>Factor (F)</u>	<u>Potassium Phosphates</u>	<u>Factor (F)</u>
Na ₂ HPO ₄	4.58	K ₂ HPO ₄	5.61
(NaPO ₃) ₆	3.29	—	—
Na ₅ P ₃ O ₁₀	3.96	K ₅ P ₃ O ₁₀	4.82
Na ₄ P ₂ O ₇	4.29	K ₄ P ₂ O ₇	5.32
NaH ₂ PO ₄	3.87	KH ₂ PO ₄	4.39
Na ₂ H ₂ P ₂ O ₇	3.58	—	—

In the event that the sodium phosphate used is not known, use the 3.96 factor to calculate added phosphate.

In the event that the potassium phosphate used is not known, use the 4.82 factor to calculate added phosphate.

In the event that a mixture of phosphate is used, use the factor for the phosphate present which will result in the highest value.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.



3.010 Automated Determination of Phosphorus

3.010A. Theory

Phosphate and Mo^{+6} react in acid solution to produce 12-molybdophosphoric acid, which is reduced with 1-amino-2-naphthol-4-sulfonic acid to phosphomolybdenum blue. Maximum absorbance at 660 nm is proportional to amount phosphorus present. Method is applicable to 0.05-0.4 percent phosphorus.

This determination can be run simultaneously with the nitrogen determination. Therefore, the apparatus and some reagents are common to both the nitrogen and the phosphorus determinations.

3.010B. Apparatus

(a) Automatic analyzer: Use the nitrogen input manifold and the phosphorus analytical manifold (Figure 1) using 660 nm filters.

(b) See §3.004B Apparatus.

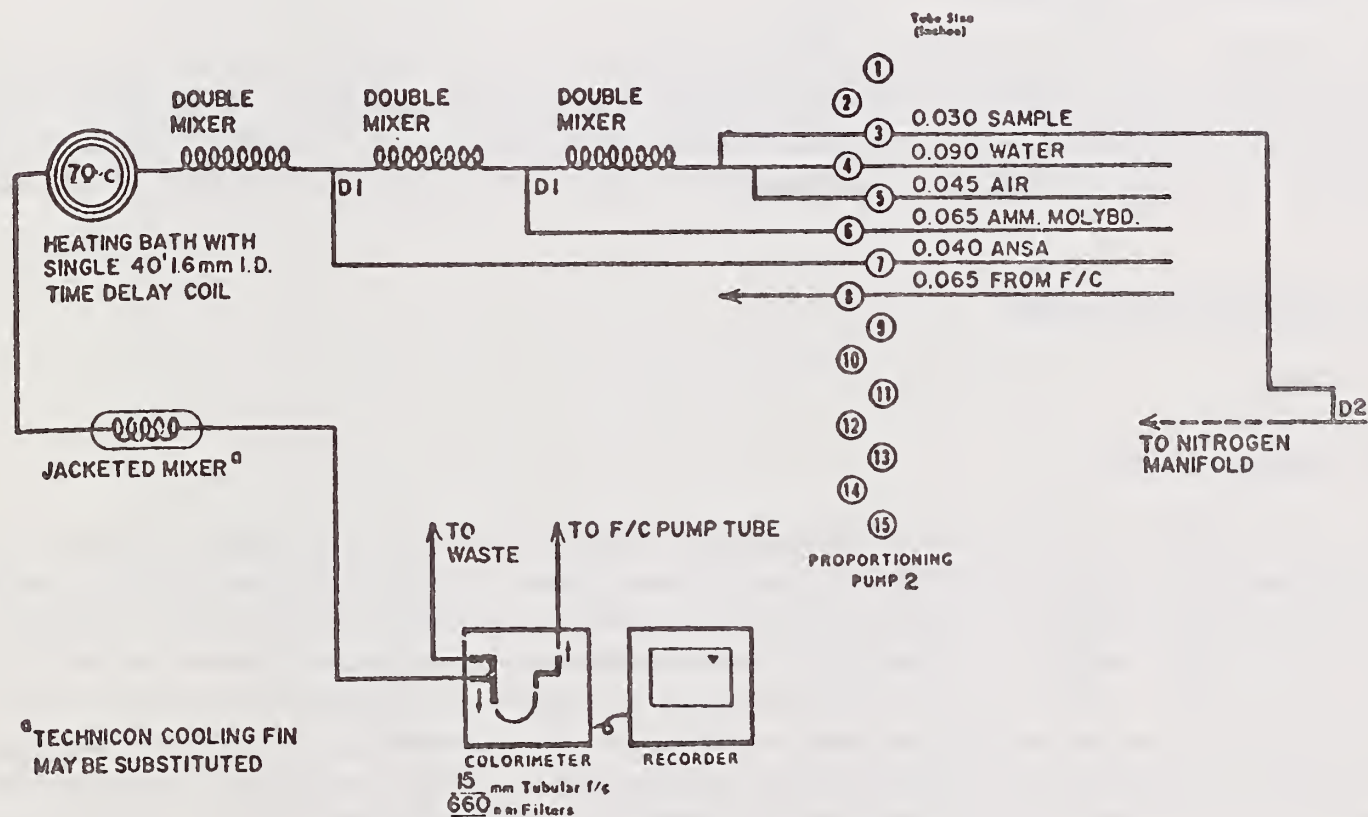


Figure 1.—Phosphorus analytical manifold.

3.010C. Reagents

(a) See §3.004C(a), (b), and (c).

(b) 1-Amino-2-naphthol-4-sulfonic acid (ANSA).

(1) Solution A: Add 2.0 g Na_2SO_3 and 60 g NaHSO_3 to 320 ml H_2O in 500 ml volume flask. Heat to 50° and add 1 g ANSA. Dissolve, cool, dilute to volume and mix. Store in amber bottle: discard when precipitate forms.

(2) Solution B: Dilute 100 ml Solution A to 1 liter with H_2O . Add 0.5 ml Levor IV wetting agent (slurry containing 40 percent sodium nonylbenzene sulfonate, Technicon Instruments Corp.). Store in amber bottle. Refrigerate when not in use. Rate of consumption is 36 ml/hr.

(c) Ammonium molybdate solution: Dissolve 30 g $(\text{NH}_4)_6\text{MO}_7\text{O}_{24}\text{H}_2\cdot 4\text{H}_2\text{O}$ in ca 1 L H_2O . Dilute to 2 L and mix. Rate of consumption is 96 ml/hr.

(d) Dilution water.

(1) Pumped through A7 fitting (Figure 1 of 3.004): Rate of consumption is 468 ml/hr.

(2) Pumped through phosphorus analytical manifold (Figure 1 of 3.010B): Rate of consumption is 174 ml/hr.

3.010D. Preparation of Standard

Weight 10.9839 g KH_2PO_4 into 250 ml volume flask, add H_2O to dissolve, and dilute to volume. Transfer 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0, and 40.0 ml to 8 separate 1 liter volumetric flasks. Add H_2O to 500 ml. Place flasks in ice bath and slowly add 500 ml H_2SO_4 to each. Cool, dilute to volume with H_2O , and store in 1 liter polyethylene bottles. Based on 10 g sample, as prepared in §4.010D, percent phosphorus = 0.050, 0.075, 0.100, 0.150, 0.200, 0.250, 0.300, and 0.400.

3.010E. Preparation of Sample

See §3.004E.

3.010F. Analytical System

Use standard 0.0625 inch id transmission tubing throughout system unless otherwise specified. Pump sample at 2.5 ml/min and segment with air pumped at 0.8 ml/min. Pump digestion mixture at 8.28 ml/min through PCl fitting and add to sample at A7 fitting. Pass sample stream through 14-turn mixing coil into inlet of digester helix. Pump dilution H_2O at 7.80 ml/min through A7 fitting to outlet end of digester helix. Aspirate diluted sample into bubble chamber and remove aliquot for analysis at 0.35 ml/min. Dilute aliquot with H_2O pumped at 2.90 ml/min and segment with air at 0.80 ml/min. Pass stream through 28-turn mixer, and add NH_4 molybdate at 1.60 ml/min. Pass stream through second 28-turn mixer followed by additional of ANSA Solution B pumped at 0.60 ml/min. After final 28-turn mixer, pass stream into 70° heating bath for color development, cool in jacketed mixer, and pass into chlorimeter equipped with 660 nm filters and 15 mm tubular flowcell. Measure absorbance at 660 nm. Pump stream from flowcell at 1.60 ml/min.

3.010G Start-up Procedure

See §3.004G.

3.010H. Determination

(a) Proceed as in §3.004H., steps (a), (b), and (c).

(b) Read absorbance of samples from recorder strip chart and compare with standard curves of absorbance against % phosphorus on 1 cycle, 70 division semilog paper. A strip chart paper may also be used.

(c) Include standard curve with every 30 samples. (percent phosphorus can be converted to percent Na tri-polyphosphate, using gravimetric factor 3.96, after percent phosphorus naturally occurring in meat is deducted.) Dilution error caused by variation in moisture content of samples does not significantly affect phosphorus determination. Standard curve is linear through range of standards.

3.010I. Shut-Down Procedure

Proceed as in §3.004I.

3.011 Phosphate Determination in Pickle Solutions

In this procedure, a weighed aliquot is hydrolyzed to convert all phosphates to the ortho form, and separated as quinolinium phosphomolybdate.

3.011A. Theory (See 3.006A.)

3.011B. Apparatus

- (a) Gooch crucible: Coors No. 4
- (b) Glass fiber filter paper: 2.4cm circles
- (c) 200ml Kohlrausch or sugar flask
- (d) 500ml beaker and watch glass
- (e) Hood
- (f) Hot plate

Preparation of the crucible: Place Gooch crucible containing a glass fiber filter disk in suction apparatus. Center the disk and wash with approximately 50ml H_2O . Dry crucible at 250°C for 30 minutes in a forced draft oven, cool in desiccator and weigh.

3.011C. Reagents

- (a) Concentrated HCl
- (b) Concentrated HNO_3

(c) Quimociac Reagent: Dissolve 70 grams sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in 150ml H_2O . Dissolve 60 grams citric acid monohydrate in a mixture of 85ml of concentrated HNO_3 and 150ml H_2O and cool. Gradually add the molybdate solution to the citric-nitric acid solution while stirring. Dissolve 5ml synthetic quinoline, with stirring, in a mixture of 35ml of concentrated HNO_3 and 100ml H_2O . Gradually add this solution to the molybdic-nitric acid solution, mix well, and let stand for 24 hours. Filter, add 280ml acetone, dilute to 1 liter with H_2O , and mix. Store in either a non-colored polyethylene bottle or a dark brown glass bottle.

3.011D. Determination

- (a) Weigh 2.0g pickle solution into a 200ml Kohlrausch or sugar flask.
- (b) Add 5 ml concentrated HCl and 50ml concentrated HNO_3 . At this point, run a reagent blank a parallel, using the same amounts of these two acids.
- (c) In a hood, place flask on hot plate and digest the sample until approximately 15ml of solution remains.
- (d) Cool flask in hood; make to volume with distilled water. Mix thoroughly.
- (e) Filter through filter paper, (an aliquot may be taken at this step depending on estimated concentration of phosphate in pickle sample), into a 500ml beaker. Add 75ml distilled H_2O .
- (f) Add 50 ml of quimociac reagent, cover with watch glass, and boil for one minute (do not use an open flame).

(g) Cool to room temperature while swirling carefully; transfer the precipitate to the prepared crucible, and wash five times with 25ml portions of distilled H₂O, allowing each portion to drain thoroughly (use suction) before adding the next portions.

(h) Dry crucible and contents for 30 minutes at 250°C, cool in desiccator and weigh.

3.011E. Calculations

$$\text{Phosphorus Content} = \frac{[(100)(A - B)(0.014)]}{C}$$

Where:

A = Weight of sample precipitate

B = Weight of blank precipitate

C = Sample weight

0.014 = Gravimetric factor derived from:

$$\text{Atomic Weight of Phosphorus} = 30.97$$

$$\text{Molecular Weight of (QPM)} = 2212.7 = (\text{C}_9\text{H}_7\text{N})_3\text{H}_3\text{PO}_4 \cdot 12\text{MoO}_3$$

$$\frac{\text{P}}{\text{QPM}} = 0.014$$

$$\text{Phosphate content} = (\text{Phosphorus Content}) (F)$$

$$F = \frac{\text{anhydrous molecular weight of desired phosphate}}{(X)(\text{atomic weight of phosphorus})}$$

Where X = number of atoms of phosphorus in one molecule of the phosphate.

The following table lists phosphates and their Corresponding factors.

<u>Sodium Phosphates</u>	<u>Factor (F)</u>	<u>Potassium Phosphates</u>	<u>Factor (F)</u>
Na ₂ HPO ₄	4.58	K ₂ HPO ₄	5.61
(NaPO ₃) ₆	3.29	—	—
Na ₅ P ₃ O ₁₀	3.96	K ₅ P ₃ O ₁₀	4.82
Na ₄ P ₂ O ₇	4.29	K ₄ P ₂ O ₇	5.32
NaH ₂ PO ₄	3.87	KH ₂ PO ₄	4.39
Na ₂ H ₂ P ₂ O ₇	3.58	—	—

In the event that the sodium phosphate used is not known, use the 3.96 factor.

In the event that the potassium phosphate used is not known, use the 4.82 factor.

In the event that a mixture of phosphates is used, use the factor for the phosphate present which will result in the highest value.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

3.012 Cereal Determination

Cereal is added to meat food products as a binder. In this procedure, the cereal starch is dissolved in 1 + 1 HCl, precipitated, and determined gravimetrically. A rapid semiquantitative method is also described.

3.012A. Theory

As in the soybean flour and soy protein concentrate determinations, the meat is rendered soluble by treatment with an alcoholic solution of caustic potash; spices and cereal starch remaining as a sediment.

If a semiquantitative estimation of the cereal content is desired, this residue volume is read and a deduction allowed for spices.

A more accurate quantitation is obtained if the cereal starch is dissolved in 1 + 1 HCl, precipitated with 95 percent ethanol, dried and weighed.

3.012B. Apparatus

- (a) Centrifuge, with a 6-7/8" diameter head.
- (b) Centrifuge tubes, Goetz, 100 ml, Corning, Catalog #8220, or equivalent.
- (c) Gooch crucible.

3.012C. Reagents

- (a) 95 percent ethanol
- (b) Alcoholic caustic potash solution (8 percent): Dissolve 40 grams of KOH in 300 ml of 95 percent ethanol, and dilute to 500 ml with 95 percent ethanol.
- (c) Dilute hydrochloric acid (1 + 1): Mix 1 volume of concentrated HCl with 1 volume of distilled H₂O.

3.012D. Determination—Semiquantitative (Applicable only in the absence of soybean flour and soy protein concentrate).

- (a) Weigh 10.0 grams of sample into a 100 ml Goetz tube.
- (b) If CS, CSS, NFDM and/or CRDSM are present, extract with two successive 50 ml portions of warm, distilled H₂O; shake, centrifuge, decant, and discard the supernatant liquid after each extraction. (If CS, CSS, NFDM or CRDSM are absent, this extraction is to be omitted).
- (c) Add 50 ml of 8 percent alcoholic KOH solution, digest on steam bath for 20 minutes with occasional stirring, and dilute to 100 ml with 95 percent ethanol.
- (d) Allow to stand for 1 hour and read volume of sediment in tube.

3.012E. Calculations

Percent cereal = Volume of sediment in tube — 0.5 percent for spices, if present.

3.012F. Determination—Gravimetric

- (a) Centrifuge the 100 ml suspension for 5 minutes. Decant and discard the supernatant liquid.
- (b) Wash the residue with 25 ml of 95 percent ethanol, stirring the sediment thoroughly.

- (c) Centrifuge, decant, and discard the supernatant liquid.
- (d) Add 50 ml of 1 + 1 HCl, mix thoroughly, stopper, and shake for 1 minute.
- (e) Centrifuge at 2000 rpm for four minutes. If the supernatant liquid is not clear, then filter it through a double thickness 541 Whatman paper, or equivalent.
- (f) Transfer 25 ml of clear supernatant liquid to a 150 ml beaker containing 75 ml of 95 percent ethanol, mix well and let stand for 1 hour.
- (g) Filter through a tared gooch crucible, wash with two 25 ml portions of 95 percent ethanol, dry for 30 minutes at 75° C, and weigh.

3.012G. Calculations

$$\text{Percent cereal} = \frac{(A - B)(1.45)(100)}{\frac{C}{2}}$$

A = Weight of gooch crucible + starch

B = Weight of gooch crucible

C = Sample Weight

1.45 = Factor for converting from starch to cereal, assuming that cereals contain an average starch content of 69 percent.

Reference

1. Official Methods of Analysis of the Association of Official Analytical Chemists 14th Edition. (Semi-quantitative only).
2. Chemistry Laboratory Guidebook, 1971 (Gravimetric only)

3.013 Soybean Flour and Soy Protein Concentrate Determinations

Soybean flour (50 percent protein) and soy protein concentrate (70 percent protein) are added to meat food products as binders. This method utilizes the fact that dilute acid will dissolve the hemicelluloses of soybean flour and soy protein concentrate but will not affect the cereal flour starch. Soybean flour or soy protein concentrate can be determined, therefore, in the presence of cereal flour.

3.013A. Theory

If a meat food product is heated in an alcoholic solution of caustic potash, the fat is saponified and the protein hydrolyzed. This treatment renders the major constituents of meat (fat, protein and water) soluble in the medium. Spices, cellulose and starch (from cereal, if present) remain as sediment.

Dilute acid is then employed to dissolve the soybean flour hemicelluloses, which are subsequently re-precipitated with 95 percent ethanol and quantitated following a carefully controlled centrifugation. Quantitation is accomplished by means of empirical factors; 6.0 for soybean flour and 2.5 for soy protein concentrate.

It is imperative to adhere closely to the time and speed of centrifugation, due to the empirical nature of this determination.

3.013B. Apparatus

- (a) Centrifuge.
- (b) Centrifuge tubes, Goetz, 100 ml, Corning, Catalog #8220, or equivalent.

3.013C. Reagents

- (a) Ethanol—95 percent.
- (b) Alcoholic caustic potash solution (8 percent): Dissolve 40 grams of KOH in 300 ml of 95 percent ethanol, and dilute to 500 ml with 95 percent ethanol.
- (c) Dilute hydrochloric acid - 1 + 3: Mix 1 volume of concentrated HCl with 3 volumes of distilled H₂O.

3.013D. Determination

- (a) Weigh 10.0 grams of sample into a 100 ml Goetz tube.
- (b) If CS, CSS, NFDM and/or CRDSM are present, extract with two successive 50 ml portions of warm, distilled H₂O: shake, centrifuge, decant, and discard the supernatant liquid after each extraction. (If CS, CSS, NFDM or CRDSM are absent, this extraction is to be omitted).
- (c) Add 50 ml of 8 percent alcoholic KOH solution and digest on steam bath for 20 minutes with occasional stirring.
- (d) Shake well and centrifuge for 4 minutes. Decant and discard supernatant solution.
- (e) Wash residue with 25 ml of 95 percent ethanol, stirring sediment thoroughly. Centrifuge and decant, discarding alcoholic solution.
- (f) Add 50 ml of 1 + 3 HCl, mix thoroughly, stopper, and shake for 1 minute. Centrifuge at 2000 rpm for 4 minutes. (Retain residue for cereal determination).
- (g) If supernatant is not clear, filter it through a double thickness #541 Whatman paper or equivalent.

(h) Transfer 25 ml of clear supernatant to a second Goetz tube containing 75 ml of 95 percent ethanol, shake well, and allow to stand for 1 hour.

(i) Centrifuge for exactly 2 minutes at a speed (rpm) that will deliver a "g force", at the furthest inside tip of the tube, equivalent to 480, accelerating to that speed in 1 minute.

$$g \text{ force} = (1.118 \times 10^{-5}) (\text{radius of rotation in centimeters}) (\text{Speed})^2$$

radius of rotation = distance from center shaft to the furthest inside tip of tube.

Derivation

$$f_{\text{cm/sec}^2} = \text{centrifugal field as cm/sec}^2 = X\omega^2$$

X = radius in cm

ω = speed radians/sec

$$1 \text{ revolution} = 2\pi \text{ radians}$$

$$\text{so, rpm} \frac{2\pi}{60} = \frac{\text{radians}}{\text{sec}} = (.10472)(\text{rpm})$$

$$\text{and squaring, } (\text{radians/sec})^2 = (.010966)(\text{rpm})^2$$

$$f_{\text{cm/sec}^2} = (X)(.010966)(\text{rpm})^2$$

$$g = 980.6 \text{ cm/sec}^2$$

$$\text{so, } g = \frac{(X)(.010966)(\text{rpm})^2}{980.6} = (1.118 \times 10^{-5})(X)(\text{rpm})^2$$

3.013E. Calculations

$$\text{Percent Soybean Flour} = \text{Volume of sediment} \times 6$$

$$\text{Percent Soy Protein Concentrate} = \text{Volume of sediment} \times 2.5$$

NOTE: 1. The sediment remaining from the dilute HCl leaching may be used to determine cereal content, if present. Decant the HCl, mix residue with 50 ml of 1:1 HCl, and proceed as under gravimetric section of Cereal Determination.

2. See Section 3.014.

3.014 Microscopic Qualitative Identification of Soy Flour and Concentrate

3.014A. Theory

The presence of characteristic “hour-glass” or I-shaped cells (sometimes called “bearer cells”) in meat or meat food products is an indication of the presence of soya flour or concentrate in those products. Because of modern refinement techniques in the processing of soya beans for flour and soya protein concentrate, these “hour-glass” cells are often broken into small fragments which are not easily identified upon microscopic examination. This procedure, therefore, is limited in its usefulness.

3.014B. Apparatus

- (a) 250ml beaker
- (b) Glass stirring rod
- (c) Steam bath
- (d) 2 Centrifuge tubes, Goetz, 100ml, Corning #8220, or equivalent
- (e) Centrifuge
- (f) Microscope (100x), preferably equipped with polarized light accessories

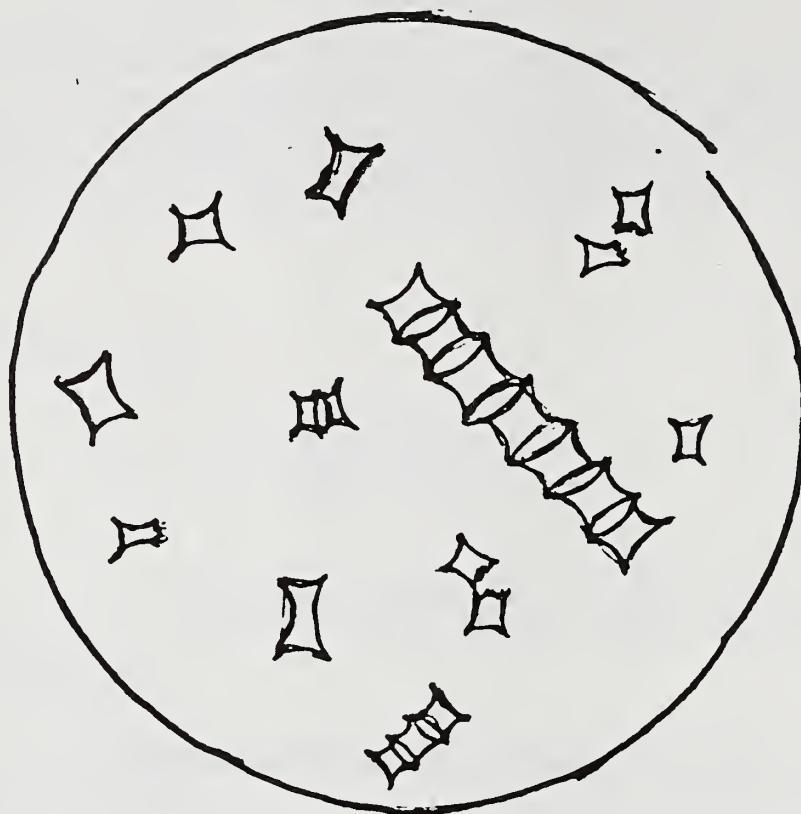
3.014C. Reagents

- (a) 8% Alc. KOH Soln.; w/v.
- (b) 95% Ethanol
- (c) Conc. HCl
- (d) 25% Ethanol in H₂O. v/v; prepared with reagent (b).

3.014D. Determination

- (a) Mix 10g finely divided samples in 250ml beaker with 75ml 8% alc. KOH soln, and heat on steam bath until all meat is dissolved (30-45 minutes).
- (b) Transfer liq. and residue to 100ml Goetz tube with 95% ethanol and let settle.
- (c) Decant as completely as possible, and cover residue with ca 50ml warm H₂O.
- (d) Stopper tube and shake vigorously; let stand a few min. until foam subsides; then transfer to a second tube and centrifuge.
- (e) Pour off and discard supernatant, and add 10ml conc. HCl.
- (f) Stopper and shake, or mix contents thoroughly with glass rod.
- (g) Add ca 15ml 25% ethanol, mix and centrifuge.
- (h) Decant supernatant and examine residue under microscope at 100x (or other appropriate magnification for characteristic “hour-glass” cells. Examination under polarized light is preferred because it causes the cells to be more visually perceptible.

Example:



NOTE: Dry powder mixtures may be examined in the same manner by wetting a small portion on a microscope slide and microscopically examining it as in step (h), above.

3.015 Lactose Determination

Nonfat dry milk (NFDM) or calcium-reduced dry skim milk (CRDSM), each consisting of approximately 50 percent lactose, is added to meat food products as a binder. In this method, the amount of added NFDM or CRDSM is determined by analyzing the product for its lactose content.

3.015A. Theory

A thorough understanding of the basic chemical principles underlying this determination helps lessen the difficulties of the analysis. We use washed baker's yeast (*Saccharomyces cerevisiae*) to ferment all reducing sugars other than lactose and maltose. If corn syrup or corn syrup solids, in addition to NFDM or CRDSM, have been added to the product, a maltose acclimated yeast must be prepared to ferment the maltose. If active dry yeast is used in lieu of baker's yeast, it is important when washing the yeast, to add the yeast to the water. The reverse procedure (adding water to the yeast) will destroy approximately 50 percent of the yeast population. Yeast consists of living, unicellular plant organisms and the dry variety must be thoroughly wet prior to stirring. It is necessary to test the yeast (as shown in a later procedure) to be sure of its viability or potency. The acclimation procedure requires care in keeping the temperature during incubation from exceeding 30°C. The motor of a mechanical stirrer, for example, could add heat to the incubation oven.

This procedure is based upon the analysis of a labile constituent of NFDM or CRDSM. Lactose can be readily oxidized to lactic acid by certain microorganisms, especially *Streptococcus lactis* which is present in muscle tissue. Lactose can also be quickly hydrolyzed to glucose and galactose by the action of hot, dilute acids. These reactions will, if they occur, cause analytical results to be low, since NFDM or CRDSM is calculated on the basis of the amount of lactose found. This analysis should be initiated as soon as the sample is ground, and, if possible, completed on the same day. The small amount of water initially added to the 20 g sample should be at room temperature when added to the sugar flask. If it is not, coagulation of the meat and milk protein might occur, making it difficult to macerate the sample and leach out the lactose.

If, following the 30-minute heating time on the steam bath, the flask and contents are not cooled to room temperature prior to adding the HCl, loss of lactose may take place by hydrolysis, as mentioned above.

To determine the viability and potency of the acclimated yeast suspension: Weigh 500 mg of maltose and 800 mg of dextrose and transfer to a 100 ml volumetric flask. Dilute to volume with distilled water, stopper, and mix well. Pipet a 10 ml aliquot into a 50 ml volumetric flask and proceed through the incubation procedure. Boiling 10 ml of this solution (following centrifugation), with 20 ml of Benedict's solution for exactly 3 minutes should yield no precipitate or suspension of Cu_2O , indicating that the yeast fermented the sugars. If a precipitate or suspension occurs, the yeast should be discarded. This is the easiest procedure for determining that the yeast is "working" properly.

NOTE: (If washed yeast is used, weigh only 800 mg of dextrose. Do not use any maltose).

The reducing portion of this method is extremely critical because it involves an empirical procedure. The 3-minute boiling time must be strictly adhered to, and the flask should be cooled rapidly following boiling, for example, invert a beaker over the neck of the flask and allow a stream of cold tap water to flow over the flask.

The titration should be performed immediately after the addition of the H_3PO_4 , to avoid any possible loss of I_2 . The use of more costly iodine flasks will also serve to avoid loss of I_2 .

3.015B. Reagents

(a) Washed yeast suspension: Mix four cakes of baker's yeast (or 30 grams of active dry yeast) to a smooth suspension with 300 ml of distilled H_2O (if active dry yeast is used, the yeast must be added to the water). Centrifuge for 5 minutes and discard aqueous layer. Repeat four more times, or until supernatant is clear following centrifugation. Finally suspend by stirring the yeast in distilled H_2O , dilute of 200 ml with distilled H_2O , and refrigerate at about 4°C.

(b) Acclimated yeast suspension: Prepare acclimating medium by dissolving each of the following ingredients in a small amount of distilled H_2O and adding, in the order given, to 1,000 ml of distilled H_2O : 2.0g anhydrous MgSO_4 , 4.0 g

NH_4Cl , 2.0 g anhydrous K_2HPO_4 , 1.0 gm KCl , 0.04 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 g peptone and 40.0 g technical maltose. Dilute to 2 liters, warm, and filter. Bring filtrate to a rolling boil and cool to room temperature.

Shake well the washed yeast suspension obtained in (a), remove 100 ml and centrifuge. Discard the aqueous layer, add the washed yeast to one liter of the acclimating medium, and incubate for approximately 24 hours of 30°C , stirring frequently the first few hours. Separate yeast by decanting and centrifuging, wash twice with distilled H_2O , and repeat incubation with the remaining one liter of acclimating medium. Separate yeast again, wash 4 or 5 times with distilled H_2O , suspend yeast in distilled water, dilute to 100 ml with distilled H_2O and refrigerate at about 4°C .

(c) Dilute hydrochloric acid: One volume conc. HCl + 4 volumes distilled H_2O .

(d) Phosphotungstic acid: 20 percent W/V.

(e) Chlorophenol red indicator: Dissolve 0.1 g chlorophenol red in 2.4 ml of 0.1N NaOH and dilute to 250 ml with distilled H_2O .

(f) Bromthymol blue indicator: Dissolve 0.1 g Bromthymol blue in 1.6 ml of 0.1N NaOH and dilute to 250 ml with distilled H_2O .

(g) Buffer solution pH 4.8: Prepare 0.1M citric acid (19.21 g/liter) and 0.2M Na_2HPO_4 (28.4 g anhydrous/liter). Mix solutions in proportions of 10.14 ml citric acid to 9.86 ml Na_2HPO_4 and adjust to pH 4.8, using a pH meter. Store in refrigerator and discard if solution becomes turbid.

(h) Benedict solution: Dissolve 16 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 150 ml of distilled H_2O . Dissolve 150 g sodium citrate dihydrate, 130 g anhydrous Na_2CO_3 and 10 g NaHCO_3 in 650 ml of distilled H_2O . Combine the two solutions, cool, dilute to 1 liter with distilled H_2O and filter.

(i) Dilute Acetic Acid: Dilute 240 ml glacial acetic acid to 1 liter with distilled H_2O .

(j) Dilute Phosphoric Acid: Dilute 240 ml phosphoric acid to 1 liter with distilled H_2O .

(k) Iodine standard solution: Dissolve 10.2 g KI in minimum quantity of distilled H_2O and use this solution as a solvent for 5.08 g I_2 . Filter, if necessary, through a glass fiber filter paper, and dilute to 1 liter with distilled H_2O .

(l) Sodium thiosulfate standard solution: Dissolve 9.92 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in recently boiled, cooled distilled H_2O , and 0.1 g Na_2CO_3 and dilute to 1 liter with distilled H_2O .

(m) Starch indicator solution: Triturate 2 g of soluble starch and 10 mg HgI_2 with a small amount of distilled H_2O . Add the suspension slowly to 500 ml boiling distilled H_2O , and boil until clear.

(n) Lactose standard solution: Dissolve 1.5789 g lactose monohydrate in distilled H_2O and dilute to 1 liter with distilled H_2O , (10 ml = 15 mg anhydrous lactose).

3.015C. Determination

(a) Weigh 20.0 g sample into a 200 ml volumetric sugar flask.

(b) Add 50 ml distilled H_2O , stir or shake to break up any lumps and heat on steam bath for 30 minutes.

(c) Cool to room temperature, add 20 ml dilute HCl , and dilute to volume using bottom of fat layer as meniscus.

(d) Add 10 ml of 20 percent phosphotungstic acid solution, mix, let stand for a few minutes, and filter through a moistened filter paper.

(e) Pipet 40 ml filtrate into a 50 ml volumetric flask.

(f) If corn syrup or corn syrup solids are absent, neutralize just to the acid side of bromthymol blue indicator, dilute to volume with distilled H_2O and mix.

(g) If corn syrup or corn syrup solids are present, neutralize just to the acid side of chlorophenol red indicator, add 5 ml of the buffer solution, dilute to volume with distilled H_2O and mix.

(h) Transfer about 40 ml of this solution to a centrifuge tube to which 5 ml of yeast suspension (washed yeast if corn syrup or corn syrup solids are absent; acclimated yeast if either one is present) has been added and from which the H_2O has been separated.

(i) Mix yeast and sample well, and incubate washed yeast for 1 hour at $30^\circ C$, or acclimated yeast for 3 hours at $30^\circ C$, stirring frequently.

(j) Centrifuge.

(k) Pipet 10 ml of clear supernatant into a 300 ml Erlenmeyer flask, add 20 ml of Benedict solution, cover with watch glass and bring to boil in 3 to 5 minutes, and boil for exactly 3 minutes.

(l) Remove from heat, cool rapidly, and add 100 ml distilled H_2O and 10 ml dilute acetic acid slowly, while swirling. Keep covered with watch glass.

(m) Add a definite volume of standard iodine solution. (15 ml for about 1.5 percent lactose, or 30 percent excess), and agitate to dissolve the Cu_2O . Keep covered with watch glass.

(n) Allow flask to stand at least 5 minutes. With watch glass just ajar, add 20 ml of dilute phosphoric acid solution. Slowly swirl to mix. Keep watch glass on. Rinse underside of watch glass into flask with distilled water before titrating excess iodine with standard sodium thiosulfate solution, using starch solution as an indicator.

(o) Determine $I_2:Na_2S_2O_3$ ratio by using 10 ml of distilled H_2O , and carrying through determination as above, beginning "... add 20 ml of Benedict solution ..."

$$I_2: Na_2S_2O_3 \text{ ratio} = \frac{\text{Volume } I_2 \text{ (in ml)}}{\text{ml } Na_2S_2O_3} = A$$

(p) Determine Lactose: I_2 ratio by using 10 ml of standard lactose solution, and carrying through determination as above, beginning "... add 20 ml of Benedict solution. ..."

$$\text{Lactose: } I_2 \text{ ratio} = \frac{15\text{mg Lactose}}{\text{ml } I_2 - (\text{ml } Na_2S_2O_3)(A)} = B$$

3.015D. Calculations

$$\text{Percent of Lactose} = \frac{100 [\text{ml of } I_2 \text{ added to flask} - (A)(\text{ml of } Na_2S_2O_3 \text{ required for back titration})] [B]}{C}$$

A & B = Ratios defined above

C = Milligrams of sample in aliquot (consider the volume of the original sample solution as 200 ml, rather than 210 ml, to take into account the volume occupied by the sample).

Percent NFDM or Percent CRDSM = (Percent Lactose \times 2) - correction

Correction: 0.4 percent in the absence of corn syrup or corn syrup solids, and 0.8 percent in the presence of corn syrup or corn syrup solids.

Note: This procedure may also be used to determine total reducing substances (total sugars calculated as dextrose) in a sample by performing the method as above, except for the following changes:

1. 3.009B Reagent(n) — Dextrose standard solution — Dissolve 1.500g dextrose in distilled water and dilute to 1 liter with distilled water (10 ml = 15 mg dextrose).
2. Add 20 ml dilute HCl at step 3.009C(b), rather than 3.009C.(c).
3. In step 3.009C.(e), dilute filtrate to 50 ml.
4. Skip steps 3.009C.(f)-(j) and proceed as in 3.009C.(k).
5. Determine the Dextrose: I₂ ratio in step 3.009C.(p) by using the dextrose standard in change 1, of this note.
6. If changes 1-5, above, are performed, total reducing sugars calculated as dextrose may be calculated in the same manner as stated in 3.009D., above, substituting the Dextrose: I₂ ratio for B in the calculation.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

3.016 Determination of NFDM (lactose) in Meat by GLC

Nonfat dry milk is added to meat and poultry processed products as a binder. The amount is limited to 3.5% (in the absence of other binders) in products which have an added water requirement.

3.016A. Theory

Lactose from the nonfat dry milk is extracted with distilled water on a steambath. The water is removed in a freeze dryer. The dry solid is silylated and quantitated by GLC, using a flame ionization detection. Lactose appears as two peaks (the d and l isomers) with retention times of approximately 4.65 and 6.35 minutes, using the GC parameters as stated in the method below.

3.016B. Apparatus

(a) Gas Chromatograph — Tracor 222, or equivalent, equipped with a flame ionization detector. Operating parameters for the Tracor 222: injector temperature, 240°C; detector temperature, 250°C; column oven, 260°C. Gas flow rates: nitrogen, 40 ml/min; hydrogen, 45 ml/min; air, 400 ml/min.

(b) GLC column — 6 ft × 4 mm ID, U-shaped; glass. Pack with 5% OV-1 on ¹⁰⁰/₁₂₀ mesh chromosorb W-HP. Condition at 280°C until stable baseline is obtained.

(c) Freeze dryer — Virtis Model 10 —145 MRBD with Model 10-MCTR cabinet, or equivalent.

(d) Scintillation vials (20 ml w/screw cap).

(e) Centrifuge — Sorvall Model RC-5, or equivalent.

(f) Culture Tubes — 16 × 100 mm (Pyrex 9825).

(g) Sugar flask, 100 ml (Kohlrausch) — (Pyrex 5780).

3.016C. Reagents

(a) Pyridine, anhydrous, ACS reagent grade.

(b) Hexamethyldisilazane, Applied Science Laboratories, State College, PA 16801.

(c) Trimethylchlorosilane, Applied Science Laboratories, State College, PA 16801.

(d) Silylation reagent: Prepare a 4:2:1 mixture of reagents (a), (b), and (c), respectively.

(e) Lactose, reagent grade.

(f) Lactose Standard: Dilute 0.100 g of lactose to 100 ml with distilled water. Transfer 2.0 ml to a scintillation vial and remove the water by freeze drying. Add 2.0 ml of silylation reagent, shake vigorously, and let stand 1 hour.

3.016D. Determination

(a) Weigh 10.0 g of ground meat into a 100 ml sugar (Kohlrausch) flask.

(b) Add ca 50 ml of water and mix to break up the lumps.

(c) Heat on a steambath for 30 minutes, mixing occasionally.

(d) Cool, dilute to volume, and filter.

- (e) Pipet, 2.0 ml of filtrate into a scintillation vial and freeze dry. (This operation may proceed overnight).
- (f) Add 2.0 ml of silylation reagent, close vial with plastic cap and shake vigorously. Let stand for 1 hour to allow reaction to reach completion. Transfer to culture tubes.
- (g) Centrifuge for 15 min @ 2000 RPM to settle the precipitate.
- (h) Inject 2.0 to 5.0 μ l of clear solution onto gas chromatographic column and compare response to that of the standard.

3.016E. Calculations

$$\% \text{ Lactose} = \frac{R_s}{R_{std}} \times \frac{V_{std} \times C_{std}}{V_s} \times \frac{DF}{W} \times 100$$

R_s = response of sample (the sum of the two lactose peak heights or peak areas)

R_{std} = Response of standard (the sum of the two lactose peak heights or peak areas)

V_s = volume of sample injected

V_{std} = volume of standard injected

C_{std} = Concentration of standard (mg/ml)

Df = dilution factor = 100, if method followed as written

W = weight of sample = 10,000 mg, if method followed as written

$$\% \text{ NFDM} = \% \text{ lactose} \times 2$$

3.017 Corn Syrup Solids Determination

Corn syrup solids (CSS) are added to meat food products as a flavoring agent. In this procedure, the amount of added CSS is determined by analyzing the product for its maltose content.

3.017A. Theory

Because recent studies have indicated that the maltose content of CSS is quite variable, it is imperative that same lot of CSS be analyzed for this constituent; this value is then used in calculating the CSS content of the product.

If corn syrup (CS) is used, a sample of the CS should be analyzed for moisture content, and only those samples which assay 20 percent or less moisture should be permitted in the product.

Samples to which CSS (or CS) and NFDM (or CRDSM) have been added, are analyzed for CSS content by determining the difference between the reducing sugars remaining in the sample after it has been subjected to two fermentations: (1) by washed yeast (which leave lactose and maltose), and (2) by yeast acclimated to maltose (which leaves lactose).

Samples to which CSS or CS, but neither NFDM nor CRDSM have been added, are analyzed for CSS content by determining the amount of maltose present following a washed yeast fermentation.

A substantial portion of the "THEORY" section of the Lactose Determination is applicable to this method.

3.017B. Reagents

Same as for Lactose Determination, except delete (n) Lactose standard solution, and insert (n) Maltose standard solution—Dissolve 1.5789 g Maltose monohydrate in distilled H₂O and dilute to liter with distilled H₂O (10 ml = 15 mg anhydrous Maltose).

3.017C. Determination

For samples containing both CSS (or CS) and NFDM (or CRDSM), proceed as under Lactose "Determination" section, pipetting two 40 ml aliquots of protein-free filtrate into 50 ml volumetric flasks.

- (a) Neutralize one just to the acid side of bromthymol blue indicator, dilute to volume with distilled H₂O and mix.
- (b) Neutralize the other just to the acid side of chorophenol red indicator, add 5 ml of the buffer solution, dilute to volume with distilled H₂O and mix.
- (c) To a centrifuge tube add 5 ml of washed yeast suspension; to another centrifuge tube add 5 ml of acclimated yeast suspension.
- (d) Separate the H₂O by centrifuging and decanting.
- (e) Transfer about 40 ml of the unbuffered solution to the centrifuge tube containing the washed yeast.
- (f) Transfer about 40 ml of the buffered solution to a centrifuge tube containing the acclimated yeast. Mix yeast and sample well. incubate washed yeast for one hour at 30°C and acclimated yeast for three hours at 30°C, stirring frequently.
- (g) Continue as under Lactose "DETERMINATION" section, except that in lieu of a Lactose: I₂ ratio, determine a Maltose: I₂ ratio using 10 ml of standard Maltose solution.

$$\text{Maltose: I}_2 \text{ ratio} = \frac{\text{mg Maltose}}{\text{ml I}_2 - (\text{ml Na}_2\text{S}_2\text{O}_3) (A)} = B$$

- (h) For samples containing either CSS or CS, but neither NFDM nor CRDSM, pipet a single 40 ml aliquot of protein-free filtrate into a 50 ml volumetric flask.

- (i) Neutralize just to the acid side of bromthymol blue indicator, dilute to volume with distilled H₂O and mix.
- (j) Transfer about 40 ml of this solution to a centrifuge tube to which 5 ml of washed yeast suspension has been added and from which the H₂O has been separated.
- (k) Mix yeast and sample well and incubate for 1 hour at 30°C. Proceed as under Lactose "DETERMINATION" section, using a Maltose:I₂ ratio rather than a Lactose:I₂ ratio.

3.017D. Calculations

For product containing CSS (or CS) and NFDM (or CRDSM)

$$\text{Percent Maltose} = \frac{100 [D - AE) - (F - AG)] [B]}{C}$$

For product containing CSS or CS, but neither NFDM nor CRDSM

$$\text{Percent Maltose} = \frac{100 [D - AE] [B]}{C}$$

A = I₂:Na₂S₂O₃ ratio

B = Maltose:I₂ ratio

C = Milligrams of sample in aliquot (consider the volume of the original sample solution as 200 ml, rather than 210 ml, to take into account the volume occupied by the sample).

D = ml of I₂ added to flask (washed yeast)

E = ml of Na₂S₂O₃ required for back titration (washed yeast)

F = ml of I₂ added to flask (acclimated yeast)

G = ml of Na₂S₂O₃ required for back titration (acclimated yeast)

$$\text{Percent CSS} = (100) \frac{\text{Percent Maltose}}{\text{Percent Maltose in CSS}} - H$$

H = Correction factor to be applied when NFDM or CRDSM is absent = 0.4 percent

No correction factor should be applied when NFDM or CRDSM is present.

Reference

Chemistry Laboratory Guidebook, 1971.

3.018 Determination of Internal Cooking Temperature

The regulations require that all processed pork products be cooked to a temperature high enough to kill trichinae. In addition, APHIS Veterinary Services requires an internal cooking temperature of 156°F on imported pork products, from certain countries, to kill the foot-and-mouth virus and other exotic viruses. The following two procedures are used to determine the maximum internal cooking temperature reached in the processing of a meat product.

The coagulation test is suitable, as a screening method, for use on all meat products for temperatures below 150°F. Above 150°F, the method is not accurate and the phosphatase procedure should be used, but only on canned picnics and canned hams, received either in the can or in a hard frozen condition.

See Section 1.002B III E for sample preparation for internal cooking temperature determinations.

3.018A. Determination of Internal Cooking Temperature by Phosphatase Activity

This method involves incubation of weighed samples with a substrate in a constant temperature bath. The released phenol is then measured spectrophotometrically.

3.018B. Theory

The enzyme (protein) acid phosphatase is denatured by heat. The activity of the phosphatase left after cooking is expressed as the amount of phenol formed, when the sample is allowed to act upon the substrate disodium phenylphosphate for a constant time, at a constant temperature and at a fixed pH. The phenol produced is reacted with 2,6-dibromoquinone chlorimide, and the absorbance of the blue color formed is measured spectrophotometrically at 610 nm.

The mechanism for the standardization of the stock phenol solution is shown by the following equations:

Potassium bromate + Potassium bromide + HCl → Bromine + Potassium chloride + Water

Phenol + Bromine → Tribromophenol hypobromite + Hydrogen bromide

Tribromophenol hypobromite + Hydrogen bromide + Potassium iodide → Tribromophenol + Potassium bromide + Iodine

The iodine is titrated with thiosulfate and the amount of phenol is calculated.

Each ml 0.1N potassium bromate = .001569 g phenol.

3.018C. Reagents

NOTE: Do not use plastic labware.

(a) Citrate buffer pH 6.5 ± 0.1: Dissolve 41.64 g trisodium citrate and 1.765 g citric acid in distilled water and dilute to 3 liters. Preserve with 3 ml toluene and store in a refrigerator.

(b) Fifty percent trichloroacetic acid: Dissolve 500 g TCA in distilled water and dilute to 1 liter.

(c) Twenty percent trichloroacetic acid: Dilute 200 ml 50 percent TCA to 500 ml with distilled water.

(d) Five percent trichloroacetic acid: Dilute 100 ml 50 percent TCA to 1 liter with distilled water.

(e) Sodium carbonate 0.5 M: Dissolve 53 g anhydrous sodium carbonate in distilled water and dilute to 1 liter.

(f) 2,6-dibromoquinone chlorimide (make fresh daily): Dissolve 40 mg 2,6-dibromoquinone chlorimide in 10 ml absolute alcohol. (Store reagent itself, in a brown bottle in a desiccator).

(g) Disodium phenyl phosphate 0.01 M: Dissolve 0.436 g disodium phenyl phosphate in distilled water and dilute to 200 ml (prepare immediately before use).

(h) Stock phenol solution: Dissolve 1.000 g phenol in distilled water and dilute to 1 liter.

(i) Working phenol solution: Transfer 5 ml of stock phenol solution (h) to 1 liter volumetric flask. Add 100 ml 50 percent TCA and dilute to volume with distilled water, shake well.

The following reagents are for standardization of stock phenol solution:

(j) Starch indicator (prepared solutions can be purchased): Mix 1 g soluble starch with 5 ml water. Add to 95 ml boiling water. Mix, cool, filter, add 0.01 g HgI_2 .

(k) Potassium bromate 0.1 N,: Dry potassium bromate, at 100°C for 2 hours, cool and store in a desicator. Weigh 2.783 g and dilute to 1 liter with distilled water. Standardize using NBS arsenious oxide as stated in "Official Methods of Analysis of the Association of Official Analytical Chemists," 14th Edition Sections 50.005-50.006 and 50.020-50.021.

(l) Sodium thiosulfate 0.1N.: Dissolve 25 g sodium thiosulfate pentahydrate and 0.2 g sodium carbonate and dilute to 1 liter with freshly boiled distilled water.

(m) Potassium iodide 10 percent: Dissolve 5 g KI and dilute to 50 ml with distilled water.

(n) Sulfuric acid 2N: Dilute 5.6 ml concentrated H_2SO_4 to 100 ml with distilled water.

(o) Hydrochloric acid 2 N: Dilute 17.8 ml concentrated HCL to 100 ml with distilled water.

(p) Potassium bromide: Reagent Grade.

3.018D. Apparatus

NOTE: Do not use plastic labware:

(a) 50 ml centrifuge tubes (Pyrex 8424 or equivalent).

(b) 15 ml test tubes.

(c) Constant temperature waterbath (37°C).

(d) Spectrophotometer - suitable for reading at 610 nm.

(e) Pipettes—various sizes.

(f) Stopwatch.

(g) Syringe - 100 μl

3.018E. Determination (avoid phenolic plastic labware)

(a) Weigh 2.50 g sample into each of four glass stoppered test tubes (A, B, C, and D). Tubes A and B are to be used for duplicated determinations. Tubes C and D are duplicate control samples.

(b) Pipet 10 ml citrate buffer into each tube.

(c) Pipet 5 ml 20 percent TCA into control samples C and D only.

(d) Stopper and shake well.

- (e) Place all tubes in a water bath at $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 10 minutes before proceeding.
- (f) Pipet 5 ml disodium phenyl phosphate solution into each tube in turn at exactly 60-second intervals using a stop watch.
- (g) Shake all tubes at 10-minute intervals.
- (h) After exactly 60 minutes, by stopwatch, pipet 5 ml 20 percent TCA to each tube in turn at 60 second intervals *except tubes C and D*. (Tube A at 60 minutes, tube B at 61 minutes, etc.).
- (i) Remove each tube from water bath after addition of TCA, shake well and filter twice through whatman 2V filter paper (Filtrates may be stored at 4°C if needed.)
- (j) Pipet 3 ml clear filtrates into clean test tubes.
- (k) Pipet 3 ml sodium carbonate into each tube. Swirl to mix.
- (l) Add 100 μl 2,6-dibromoquinone chlorimide solution into each tube using an 100 μl syringe, mix well by swirling.
- (m) Develop color in the dark for at least 30 minutes (not overnight). If tube C is blue, contamination has occurred. Begin test again, using new 2.5 g samples and clean labware.
- (n) Read absorbances of each solution at 610 nm using 1 cm cells and water as reference for setting the spectrophotometer at 100 percent transmission. Spectrophotometer should be calibrated with a holmium oxide crystal.

3.018F. Standards

- (a) Preparation of Standards
 - (1) Pipet duplicate aliquots of 0.0 ml, 0.5 ml, 1.0 ml and 2.0 ml of working phenol solution into test tubes (8 tubes total).
 - (2) Pipet 5.0 ml, 4.5 ml, 4.0 and 3.0 ml respectively, of 5 percent TCA making each tube equal in volume (5ml).
 - (3) Add 5.0 ml, 0.5 M sodium carbonate.
 - (4) Pipet 0.1 ml 2,6-dibromoquinone chlorimide into each tube.
 - (5) Swirl and develop color for at least 30 minutes in the dark.
 - (6) Measure the absorbance of each tube of 610 nm, using 1 cm cells and water as reference for setting spectrophotometer at 100 percent transmission.
- (b) Determination of sodium thiosulfate factor.
 - (1) Add 2 g KI, 25 ml 0.1 N potassium bromate and 20 ml 2 N HCL in an Erlenmeyer flask.
 - (2) Let stand 15 minutes in the dark.
 - (3) Slowly add 150 ml distilled water.
 - (4) With steady swirling or on magnetic stirrer, titrate with 0.1 N sodium thiosulfate until the disappearance of the blue color, using 1 ml of starch solution as indicator.

(c) Determination of phenol concentration in stock phenol solution:

(1) Pipet 50.0 ml stock phenol solution into glass stoppered 500 ml Erlenmeyer flask.

(2) Pipet 50.0 ml 0.1 N potassium bromate solution into the flask.

(3) Add 2.0 g potassium bromide.

(4) When the latter has dissolved add 20 ml 2 N H_2SO_4 , mix.

(5) Let stoppered solution sit for 15 minutes in the dark.

(6) Carefully pipet 10 ml 10 percent KI into mixture removing stopper as little as possible.

(7) Shake well and let sit 15 minutes in the dark.

(8) Titrate with 0.1 N sodium thiosulfate, after rinsing stopper and sides of flask with distilled water, adding 1 ml starch indicator. End point is indicated by the absence of blue color from the gel particles. A bright light and white background may be necessary.

3.018G. Calculations

$$(a) \text{ N As}_2\text{O}_3 = g \text{ As}_2\text{O}_3 \times 4000/\text{ml final volume} \times 197.84$$

$$A = 0.1 \text{ N potassium bromate factor} = \text{ml As}_2\text{O}_3 \times \text{normality As}_2\text{O}_3/\text{ml kBr} - \text{kBrO}_3$$

$$(b) B = 0.1 \text{ N sodium thiosulfate factor} = \frac{25.00 \times A}{\text{ml C}}$$

$$C = \text{ml thiosulfate titrated in step 3.011F(b)(4)}$$

$$(c) \% \text{ Phenol} = \frac{[(50)(A) - (B)(D)] 0.1569}{50}$$

$$D = \text{ml thiosulfate titrated in step 3.011F(c)(8)}$$

$$(d) X = \text{mg Phenol}/100 \text{ ml stock solution}$$

$$(e) Y = \text{u moles phenol}/1000 \text{ ml working phenol solution} = \frac{(X)(5)(1)}{94.11}$$

Molecular wt. of phenol = 94.11

$$(f) \text{ Extinction} = \frac{(\text{absorbance tube A} + \text{absorbance tube B})}{2} - \frac{\text{absorbance tube C} + \text{absorbance tube D}}{2}$$

Read absorbance to the nearest 0.001 absorbance unit.

After correcting for the blank (abs of the 0.0 ml standard substituted in the above formula in place of abs tubes C and D) the extinction values of the standard solutions are treated to determine the standard factor F.

$$(g) F 0.5 \text{ standard} = \frac{0.5Y}{\text{extinction}} = \text{u mol phenol}/\text{extinction unit}$$

$$(h) \text{ F 1.0 standard} = \frac{Y}{\text{extinction}} = \text{u mol phenol/extinction unit}$$

$$(i) \text{ F 2.0 standard} = \frac{2Y}{\text{extinction}} = \text{u moles phenol/extinction unit}$$

$$(j) \text{ F} = \frac{\text{F 0.5 standard} + \text{F 1.0 standard} + \text{F 2.0 standard}}{3}$$

To evaluate the phosphatase activity of the sample:

$$(k) \text{ EF}' = \text{u moles phenol/1000 g sample} = \frac{(F)(\text{Sample Extinction})(1000)}{62.5^*}$$

$$(l) \text{ }^{\circ}\text{C Internal Cooking Temperature} = 77.3985 - (5.7109)(\text{Log EF}')$$

$$(m) \text{ }^{\circ}\text{F} = \frac{9}{5} \text{ }^{\circ}\text{C} + 32$$

The formula used to calculate the internal temperature was derived empirically. It may be necessary to redetermine the formula if processing procedures are changed.

- NOTE: 1. Correction for products with salt content higher than 3.55%. Add 0.95° F for each 1% above 3.55%.
2. Repeat analysis if the following criteria are not met:

Abs of tubes A & B between	Acceptable abs difference between tubes A and B
0.35 to 0.50	0.035
0.20 to 0.34	0.028
0.10 to 0.19	0.020
Less than 0.10	0.016

Reference

Lind, J., Determination of Activity of Acid Phosphatase in Canned Hams, Danish Meat Products, Laboratory, The Royal Veterinary and Agriculture College, September 23, 1965.

*62.5 = the dilution factor used to convert g/ml to micro moles phenol/1000 g sample.

$$\frac{2.5 \text{ g}}{20 \text{ ml}} \times \frac{3 \text{ ml}}{6 \text{ ml}} \times 1000$$

3.019 Determination of Internal Cooking Temperature (Coagulation)

Soluble proteins are extracted with a 0.9 percent saline solution, subjected to heat, causing a cloudiness or turbidity in the extract because of coagulated proteins. The temperature at which the first sign of cloudiness appears is the maximum internal cooking temperature. This procedure is empirical and must be followed as written.

This procedure is applicable to both beef and pork products below 150°F., that do not contain any organ meats or nonmeat proteins.

3.019A. Reagents

0.9 percent saline solution: dissolve 9.0 of sodium chloride in 200 ml of H₂O. Make to one liter; mix thoroughly.

3.019B. Apparatus:

- (a) Gooch Crucible
- (b) Celite 545
- (c) Buchner funnel
- (d) Filtering flask with tubulation
- (e) Filter paper—suitable for use in Buchner funnel
- (f) Test tube—50 ml
- (g) Water bath—consisting of 1 to 2 liter beaker with a stirring apparatus and clamps or rack for holding test tubes
- (h) Bunsen burner or equivalent
- (i) Two thermometers

3.019C. Determination:

- (a) Weigh 50 g of ground sample into a 250 ml beaker
- (b) Add 100 ml of saline solution; thoroughly mix and allow to stand for 20 min.
- (c) Filter the mixture through a filter paper on a Buchner funnel using vacuum.
- (d) Filter the filtrate a second time using a Gooch crucible and a coarse filter paper with a Celite pad of appropriate thickness.
- (e) The second filtrate should be nearly clear; if not repeat step (d). NOTE: if filtrate is too clear all protein may have been removed.
- (f) The filtrate is divided into two test tubes. One is the control; the other the test portion.
- (g) The test sample is clamped into position in the water bath and a thermometer is suspended in the filtrate. A second thermometer is suspended in the water.
- (h) Apply heat to the water being careful to maintain a difference $\leq 1.5^{\circ}\text{F}$ between the water temperature and the temperature of the filtrate.

(i) The temperature at which the first sign of cloudiness appears is the maximum internal cooking temperature, and continue to heat up to 156°F. If a definite cloudiness does not appear, add ca 1 ml 20% phosphotungstic acid to confirm presence of protein. If no cloudiness appears, start over.

NOTE: For rapid screening, the filtrate may be immersed in a water bath at 156°F. Those samples that appear to cloud may be removed and a fresh aliquot treated to slower temperature rise to determine the maximum cooking temperature.

Reference

USDA, Food Safety and Inspection Service, Chemistry Division, unpublished method.

3.020 Determination of Moisture Absorption in Giblet Wrap

The amount of moisture absorbed by giblet paper is such that (1) the wet weight of the paper shall not exceed 90 pounds per ream, and (2) the amount of moisture absorbed shall not exceed 200 percent of the dry weight of the paper.

3.020A. Apparatus:

- (a) 2 × 4 inch stainless steel die
- (b) analytical balance
- (c) scalpel

3.020B. Determination:

- (a) Select ten representative sheets of paper from the sample.
- (b) Using the 2 × 4-inch die, cut 2 × 4 inch strips from each sheet.
- (c) Determine the dry weight of each 2 × 4 inch strip using an analytical balance.
- (d) Immerse each strip in a beaker of water for one minute.
- (e) Remove the strip from the beaker and shake lightly to remove excess water.
- (f) Fold the strip lengthwise twice to reduce evaporation. *Do Not Squeeze.*
- (g) Weigh each folded strip.

3.020C. Calculations:

- (a) Determine the average weight of the dry strips.
- (b) Determine the average weight of the wet strips.

(c) Percent moisture absorbed $\frac{A - B}{B} \times 100$

A = average weight of wet strips.

B = average weight of dry strips.

(d) Wet weight per ream = $\left(\frac{\text{Percent moisture absorbed (C)}}{100} \right) + C$

Where C = Dry weight per ream of paper being tested obtained from: (Extrapolate if necessary.)

If <u>B</u> equals	then <u>C</u> equals
0.2856 g.	34 lbs.
0.2771	33
0.2687	32
0.2604	31
0.2520	30
0.2436	29
0.2352	28
0.2268	27

Reference

Technical Association of the Pulp and Paper Industry (T.A.P.P.I.), Standards T-410 and T-402

3.021 Determination of pH of Meat

3.021A. Apparatus

- (a) Beaker, 100 ml
- (b) Fluted filter paper
- (c) Glass stirring rod
- (d) pH meter, suitable for reading pH from 0 to 14 in 0.1 increments.

3.021B. Reagents

- (a) Distilled water
- (b) Certified Buffer Solutions (Commercially available or prepare as in 14th Edition AOAC, 50.008-50.010)

3.021C. Determination

- (a) With a glass stirring rod, slurry 25-50 grams of ground sample in a 100 ml beaker with an equal weight of distilled water.
- (b) Force a fluted filter paper part way down into the slurry and let set five minutes.
- (c) Calibrate the pH meter according to manufacturer's instructions, using reagent (b), at the pH closest to that expected in the sample.
- (d) Immerse pH electrodes into the filtered solution inside the fluted filter paper.
- (e) Record pH to the nearest 0.1.

PART 4—MICROANALYTICAL PROCEDURES

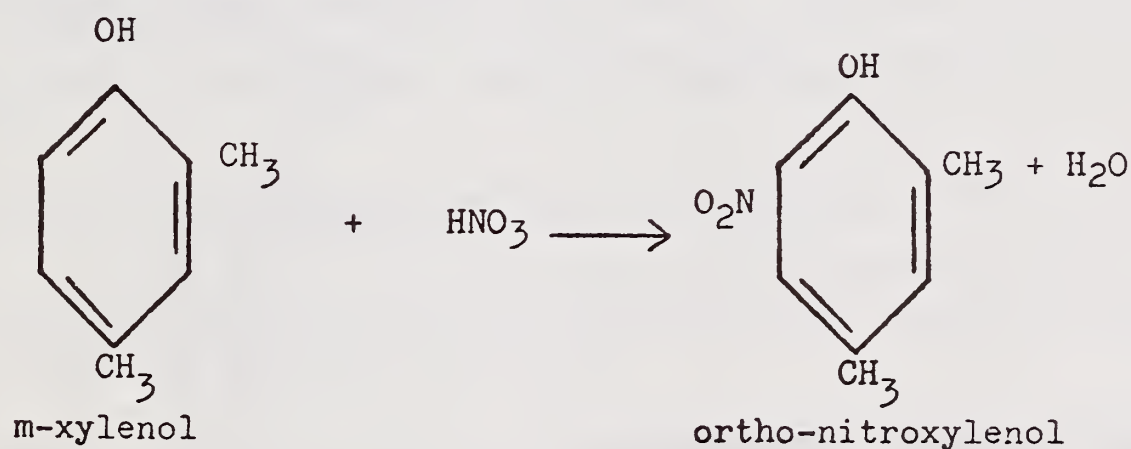
4.001 Determination of Nitrates in Meat

In this method, nitrite and nitrate are determined as total nitrate, and the actual nitrate content calculated by difference.

4.001A. Theory

This procedure is based on the fact that nitrates yield nitric acid upon treatment with sulfuric acid. The nitric acid liberated is employed to nitrate a xylenol. If, upon nitration, the incoming nitro group goes ortho to the hydroxyl group, the ortho-nitroxymenol formed will be steam distillable. The nitroxymenol is then steam distilled, and the distillate collected in an aqueous solution of NaOH, forming a colored sodium salt which follows Beer's Law.

Because there are a number of isomers of xylenol (dimethyl phenol), m-xymenol was chosen as a reagent in this analysis as it will yield only one mono-nitro derivative upon nitration. Furthermore, this derivative will be ortho-nitroxymenol, due to the strong ortho-para orientation of the OH group.



The six (6) position on the benzene ring is the one at which nitration occurs, since the 2 and 4 positions (ortho and para to the OH group) are occupied by CH₃ radicals. Nitration at the 6 position forms ortho-nitroxymenol which is steam-distillable.

Nitrites, chlorides and proteins interfere and must be removed. Nitrite is converted to nitrate by oxidation with KMnO₄; chlorides are precipitated as the silver salt, and proteins brought down with phosphotungstic acid.

4.001B. Apparatus

All glass distillation apparatus with an elongated delivery tube (see figure 1).

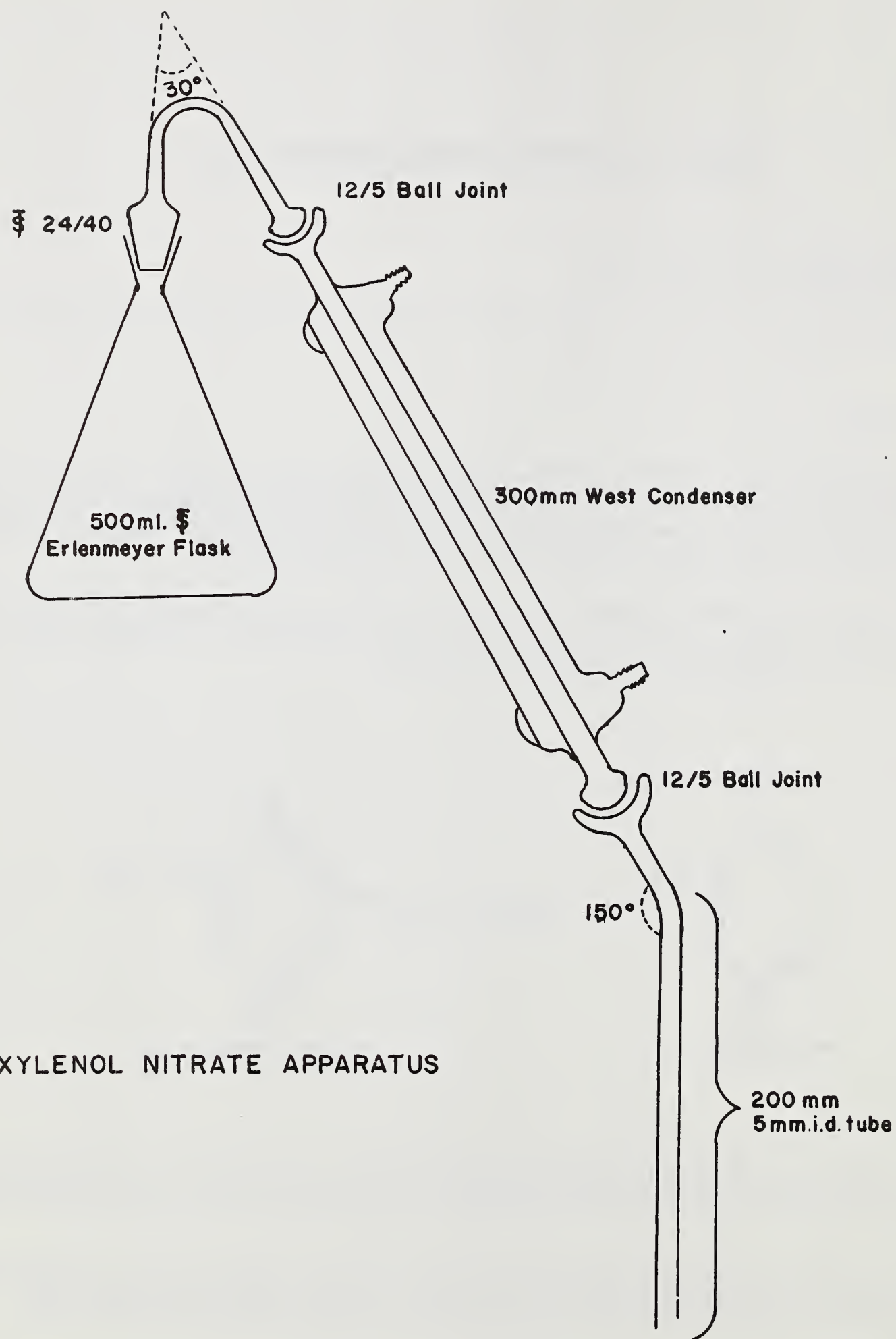


Figure 1

4.001C. Reagents

- (a) Sulfuric Acid: one volume concentrated reagent grade H_2SO_4 + 10 volumes H_2O .
- (b) Sulfuric Acid: three volumes concentrated reagent grade H_2SO_4 + 1 volume H_2O .
- (c) Potassium Permanganate 0.2N: dissolve 0.63 g KMnO_4 in H_2O and dilute to 100 ml.
- (d) Phosphotungstic Acid Solution: 20 percent w/v.
- (e) Silver-Ammonium Hydroxide Solution: dissolve 5 g nitrate-free Ag_2SO_4 in 60 ml NH_4OH . Heat to boiling, concentrate to approximately 30 ml, cool, and dilute to 100 ml with H_2O .
- (f) m-Xylenol - (2, 4-dimethylphenol): Eastman No. 1150 or equivalent.
- (g) Sodium Hydroxide Solution: one percent w/v
- (h) Bromocresol green indicator: dissolve 0.1 g bromocresol green in 1.5 ml 0.1 N NaOH, and dilute to 100 ml with H_2O .

4.001D. Determination

- (a) Weigh 10.0 g sample into a 100 ml sugar flask. Add 80 ml distilled H_2O , stir or shake to break up any lumps, and heat on steam bath for 1 hour.
- (b) Cool, dilute to volume using bottom of fat layer as meniscus, mix well and filter.
- (c) Pipet 40 ml filtrate into a 50 ml volumetric flask. At this point, add 40 ml of distilled H_2O to a 50 ml volumetric flask, and carry through the procedure as a reagent blank.
- (d) Add 3 drops of bromocresol green indicator and dilute H_2SO_4 (1 + 10) dropwise until color changes to yellow.
- (e) To assure complete oxidation of nitrite to nitrate, add 0.2N KMnO_4 dropwise with shaking until permanent pink color is obtained.
- (f) Add 1 ml dilute H_2SO_4 (1 + 10), 1 ml phosphotungstic acid solution, dilute to mark with H_2O , mix well and filter.
- (g) Pipet 10 ml filtrate into a 500 ml Erlenmeyer flask and add sufficient Ag- NH_4OH solution dropwise to precipitate all chlorides and most of the excess phosphotungstic acid. (A slight excess of the Ag reagent is not harmful). Placing the flask upon a black surface will help determine the end point.
- (h) Add 45 ml H_2SO_4 (3 + 1), stopper flask, mix, cool to approximately 35°C, add 3 drops of m-xylenol, stopper, mix thoroughly and maintain it at 30 — 40° C for 30 minutes.
- (i) After nitration is complete, add 150 ml H_2O (wash stopper), a few glass beads and distill 40-50 ml into a 100 ml volumetric flask, placed in a beaker of water and ice, containing 5 ml of one percent NaOH solution and sufficient H_2O such that the end of the delivery tube will be submerged. Remove any nitroxylenol which may have solidified in the condenser by stopping condenser water flow and allowing condenser to become hot. Do not allow steam to bubble through the distillate.
- (j) Dilute to volume with H_2O and measure absorbance at 445 nm, setting the spectrophotometer at 100 percent transmission with reagent-blank. Obtain NaNO_3 concentration from standard curve and calculate total nitrate content.

4.001E. Standard Curve

(a) Dissolve 0.1600g dried reagent grade NaNO_3 in distilled H_2O and dilute to 1 liter. Each ml of this solution contains 160 μg of NaNO_3 .

(b) Pipet 0, 1.0, 2.5, 7.5 and 10.0 ml of this solution into separate 500 ml Erlenmeyer flasks, and add a volume of distilled H_2O to each such that a 10 ml volume is obtained.

(c) Continue as in 4.001D(h) above . . . "Add 45 ml H_2SO_4 (3 + 1), stopper flask, mix, . . ." Plot absorbance at 445 nm vs. micrograms NaNO_3 per ml.

NOTE: The standard curve as prepared above, cover NaNO_3 concentrations up to 2,000 ppm for a 10g sample diluted as stated in 4.001D. For better measurements at low concentrations (less than 50 ppm), prepare a curve using standards that bracket the concentration of the sample, that is one-half to twice the level of interest. For low concentration use 5 cm cuvettes.

4.001F. Calculations

$$\text{Total NaNO}_3 \text{ content (ppm)} = \frac{(A) (B)}{C}$$

A = Micrograms NaNO_3 per ml (from standard curve).

B = Final volume of distillate (100 ml if determination is conducted as written above).

C = Sample weight in grams represented in final volume of distillate (0.8 g if dilutions are made as written above).

In order to obtain nitrate content (due solely to NaNO_3) multiply the sodium nitrite (if present) content (ppm) by the

ratio $\frac{\text{NaNO}_3}{\text{NaNO}_2} = \frac{85}{69} = 1.23$ and subtract this product from the total nitrate content.

NaNO_3 content (ppm) = Total NaNO_3 content - (1.23) (NaNO_2 content.) If nitrate is present as potassium nitrate, multiply NaNO_3 content by the ratio $\frac{\text{KNO}_3}{\text{NaNO}_3} = \frac{101}{85} = 1.19$.

KNO_3 content (ppm) = (1.19) (NaNO_3 content).

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

4.002 Determination of Nitrites in Meat

In this procedure, residual nitrite (that which has not combined to form nitrosomyoglobin) is determined spectrophotometrically.

4.002A. Theory

An aromatic primary amine will react with an acidified solution of a nitrite to produce a diazonium salt. If this salt is then condensed or coupled with another primary aromatic amine, an aminoazo compound is formed which obeys Beer's Law.

4.002B. Reagents

- (a) Sulfanilamide solution: dissolve 0.5 g in 150 ml of 15 percent (v/v) HOAc.
- (b) N-(1-naphthyl) ethylenediamine dihydrochloride solution: Dissolve 0.2 g in 150 ml 15 percent (v/v) HOAc. Store in g-s brown glass bottle.
- (c) Filter paper: check 3-4 sheets randomly selected filter paper for nitrite contamination. Filter ca 40 ml of H₂O thru each sheet and add reagents. If positive, discard box.

4.002C. Determination

- (a) Weigh 5.0 g sample into a 400-600 ml beaker, add 300 ml of distilled H₂O, stir vigorously to break up any lumps. (For acid products, adjust pH, with pH meter, to 6.5-7.0 with 0.1N NaOH, and quantitatively transfer to a 500 ml volumetric flask.) Heat on steam bath for 2 hr with occasional stirring.
- (b) Cool to room temperature, dilute to mark with distilled H₂O, mix well and filter.
- (c) Transfer 25.0 ml of filtrate (or other suitable aliquot containing 5-50 µg NaNO₂) to a 50 ml volumetric flask, add 2.5 ml of sulfanilamide solution and mix by swirling.
- (d) Allow to stand for 5 minutes, add 2.5 ml of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled water and mix well.
- (e) Let stand for 15 minutes, transfer a portion to a cuvette and measure optical density at 540 nm setting the spectrophotometer at 0.0 absorbance with a reagent blank. Obtain NaNO₂ concentration from standard curve and calculate NaNO₂ content.

4.002D. Standard Curve

- (a) Dissolve 0.2000g dried NaNO₂ (take into account the assay of your NaNO₂ reagent) in distilled H₂O, dilute to 1 liter and mix well.
- (b) Dilute 10.0 ml of this solution to 1 liter with distilled H₂O and mix well. Each ml of the final dilution contains 2.0 µg NaNO₂. (If more convenient, a "catch weight" may be taken, provided that it approximates 0.2 g and is weighed to the nearest 0.1 mg)
- (c) Pipet 0.0, 5.0, 10.0, 20.0, and 25.0 ml of the final dilution into separate 50ml volumetric flasks, add 2.5 ml of sulfanilamide solution and mix by swirling.
- (d) Allow to stand 5 minutes, add 2.5 ml of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled H₂O and mix well.
- (e) Let stand for 15 minutes and measure absorbance @ 540 nm, setting the spectrophotometer at 0.0 absorbance with the first standard solution prepared above. Plot absorbance vs micrograms NaNO₂ per ml.

Note: The standard curve as prepared above, covers NaNO_2 concentrations up to 200 ppm for a 5 g sample diluted as stated in 4.002C. For better measurements at low concentrations (less than 50 ppm), prepare a curve using standards that bracket the concentration of the sample, that is one-half to twice the level of interest. For low concentrations use 5 cm cuvettes.

4.002E. Calculations

$$\text{NaNO}_2 \text{ content (ppm)} = \frac{(A)(B)}{C}$$

where

A = Micrograms NaNO_2 per ml (from standard curve)

B = Final volume to which an aliquot was diluted (50 ml if conducted as written above)

C = Sample weight in grams represented in final volume (0.25 grams, if a 25.0 ml aliquot was taken)

If the nitrite is present as KNO_2 multiply NaNO_2 content (ppm) by the ratio $\frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$

$$\text{KNO}_2 \text{ content (ppm)} = (1.23)(\text{NaNO}_2 \text{ content, ppm})$$

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

4.003 Determination of Nitrites in Cures and Pickles in the Presence of Ascorbates

This method is very similar to the Determination of Nitrites in Meat in which a spectrophotometric analysis is performed following diazotization and coupling.

4.003A. Theory

In an acidified solution, ascorbate and nitrite will react with each other to their mutual destruction, ascorbate being oxidized and the nitrite reduced. To minimize the effects of this reaction on nitrite determinations, a procedure is used that requires 5 minutes for diazotization and 15 minutes for coupling to obtain better than a 90 percent recovery of the nitrite present.

- (a) Sulfanilamide solution: Dissolve 0.5g in 100 ml of 1:1 HCl (v/v). Store in g-s brown, glass bottle.
- (b) N-(1-naphthyl) ethylenediamine dihydrochloride solution: Dissolve 0.1 g in 100 ml distilled water.
- (c) Barium chloride: 10 percent (w/v).

4.003C. Determination

(a) *CURES*—Weigh 20.0 g of cure into a 200 ml volumetric flask. Add 100 ml of distilled H₂O, shake to dissolve cure, dilute to volume with distilled H₂O and mix well. Dilute 3.0 ml of this solution to 1 liter with distilled H₂O and mix well. If cure contains phosphate(s) transfer 3.0 ml of the first dilution to a 1 liter volumetric flask, add 15 ml BaCl₂ solution, dilute to volume with distilled H₂O and mix. Filter through a filter paper (Reeve Angel # 802 or equivalent).

(1) If the anticipated nitrite content is less than 1 percent, transfer 10.0 ml of the second solution to a 100 ml volumetric flask.

(2) If it is between 1 to 10 percent, dilute 10.0 ml of the second solution to 100 ml with distilled H₂O, mix well, and transfer appropriate dilution of this third solution to a 100 ml volumetric flask.

For cures containing more than 10 percent nitrite, make further appropriate dilutions.

(b) *PICKLES*—Dilute 3.0 ml of pickle to 1 liter with distilled H₂O and mix well. If pickle contains phosphate(s) transfer 3.0 ml of the pickle to a 1 liter volumetric flask, add 15 ml BaCl₂ solution, dilute to volume with distilled H₂O and mix. Filter through a filter paper (Reeve Angel, #802 or equivalent.) Transfer appropriate dilution of this filtrate to a 100 ml volumetric flask.

(c) *CURES AND PICKLES*—

(1) To appropriate aliquot of cure or pickle solution contained in the final 100 ml volumetric flask, add 2.5 ml of sulfanilamide solution and mix by swirling.

(2) Allow to stand for 5 minutes, add 2.5 ml of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled H₂O and mix well.

(3) Let stand for 15 minutes and measure absorbance_{540 nm}, setting the spectrophotometer at 100 percent transmission with a reagent blank. Obtain NaNO₂ concentration from standard curve and calculate NaNO₂ content.

4.003D. Standard Curve

(a) Dissolve 0.2000 g dried NaNO₂ (take into account the assay of your NaNO₂ reagent) in distilled H₂O, dilute to 1 liter and mix well.

(b) Dilute 10.0 ml of this solution to 1 liter with distilled H₂O and mix well. Each ml of the final dilution contains 2.0 µg NaNO₂. (If more convenient, a "catch weight" may be taken, provided that it approximates 0.2 g and is weighed to the nearest 0.1 mg).

(c) Pipet 0.0, 5.0, 10.0, 20.0 and 25.0 ml of the final dilution into separate 100 ml. volumetric flasks. add 2.5 ml of sulfanilamide solution and mix by swirling.

(d) Allow to stand for 5 minutes. add 2.5 ml of N - (1-naphthyl) ethylenediamine dihydrochloride solution. dilute to volume with distilled H₂O and mix well.

(e) Let stand for 15 minutes and measure absorbance 540 nm. setting the spectrophotometer at 100 percent transmission with the first standard solution prepared above. Plot absorbance *vs* micrograms NaNO₂ per ml.

4.003E. Calculations (Cures)

$$\text{Percent NaNO}_2 = \frac{(A) (B) (10^{-1})}{C}$$

where

A = Micrograms NaNO₂ per ml (from standard curve)

B = Final volume to which aliquot was diluted (100 ml if conducted as written above)

10⁻¹ = Factor to convert μg/g to percent

C = Sample weight in grams represented in final volume

4.003F. Calculations (Pickles)

$$\text{NaNO}_2 \text{ content (lbs/100 gallons)} = \frac{(A) (B) (10^{-6}) (834.5)}{V_p}$$

where

A = Micrograms NaNO₂ per ml (from standard curve)

B = Final volume to which aliquot was diluted (100 ml if conducted as written above)

10⁻⁶ = Factor to convert μg to g

834.5 = Factor to convert g/ml to lbs/100 gallons (For derivation. see "Determination of Nitrites in Cures and Pickles in the Absence of Ascorbates").

V_p = Volume of pickle represented in final volume.

If the nitrite is present as KNO₂, multiply NaNO₂ content (lb/100 gallons) by the ratio $\frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$

KNO₂ content (lb/100 gallons) = (1.23)(NaNO₂ content, lb/100 gallons)

Reference

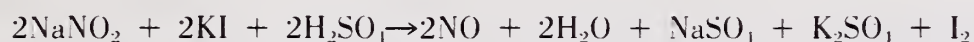
Official Methods of Analysis of the Association of Official Analytical Chemists. 14th Edition.

4.004 Determination of Nitrites in Cures and Pickles in the Absence of Ascorbates

In this procedure, nitrite is determined by reacting it with potassium iodide in acid medium.

4.004A. Theory

In acid medium, nitrite will oxidize iodide quantitatively to iodine, according to the following equation:



The liberated iodine is then titrated with sodium thiosulfate. During this reaction, nitrite is reduced to nitric oxide which will form higher oxides of nitrogen by reacting with the oxygen of the air; these higher oxides will then react with iodide to form more iodine and nitric oxide, the cycle repeating itself indefinitely, resulting in failure to attain a permanent endpoint. This induced air oxidation of iodide can be avoided by conducting the analysis in an atmosphere of carbon dioxide or any other inert gas.

4.004B. Reagents

- (a) Dilute Sulfuric Acid—1 volume of concentrated reagent grade H_2SO_4 + 9 volumes of distilled H_2O .
- (b) Potassium Iodide: 10 percent (w/v)
- (c) Carbon Dioxide: Cylinder
- (d) Starch indicator solution: Triturate 2 g of soluble starch and 10 mg H_2I_2 with a small amount of distilled H_2O . Add the suspension slowly to 500 ml boiling distilled H_2O , and boil until clear.
- (e) Sodium Thiosulfate solution: 0.05N - Dissolve 12.5 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in recently boiled, cooled, distilled H_2O , add 0.1 g Na_2CO_3 and dilute to 1 liter. Standardize against $\text{K}_2\text{Cr}_2\text{O}_7$.

4.004C. Determination

- (a) Weigh 20.0 g of cure into a 200 ml volumetric flask. Add 100 ml distilled H_2O , shake to dissolve cure, dilute to volume with distilled H_2O , stopper and mix well.
- (b) To a 300 ml Erlenmeyer flask add 20 ml distilled H_2O , 10 ml of dilute H_2SO_4 and 10 ml of 10 percent KI solution.
- (c) Bubble CO_2 gently through this solution and throughout the reaction.
- (d) Add a 10.0 ml aliquot of the cure solution (for pickles, add a 25.0 ml sample, making no dilutions) to the flask and titrate with standard thiosulfate solution using starch solution as an indicator.

NOTE: The dilution (20.0 g/200 ml) and aliquot (10.0 ml) indicated above are valid for cures containing less than 40 percent NaNO_2 . For cures of greater than 40 percent NaNO_2 content, use an appropriately larger dilution and/or a smaller aliquot.

4.004D. Calculations-(Cures)

$$\text{Percent NaNO}_2 = \frac{(V) (N) (6.9)}{S}$$

where

V = Volume of standard sodium thiosulfate solution

N = Normality of standard sodium thiosulfate solution

6.9 = meq. wt. of NaNO_2 (includes factor of 100 for percentage)

S = Sample weight in grams represented by aliquot used

If the nitrite is present as KNO_2 , multiply percent NaNO_2 by the ratio:

$$\frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$$

$$\text{Percent KNO}_2 = 1.23 (\% \text{ NaNO}_2)$$

4.004E. Calculations (Pickles)

Because the amount of nitrite which may be added to pickles is measured in pounds of nitrite per 100 gallons of pickles, the result should bear the same units.

First express the result in grams NaNO_2 per ml of pickle, and proceed from there.

$$\text{NaNO}_2 \text{ content (g/ml)} = \frac{(V) (N) (0.069)}{V_p}$$

where

V = Volume of standard sodium thiosulfate solution

N = Normality of standard sodium thiosulfate solution

0.069 = Meq. wt. of NaNO_2

V_p = Volume of pickle used

In order to convert g/ml to lbs/100 gallons, it is only necessary to multiply the former by the following factors:

$$\frac{\text{g}}{\text{ml}} \times \frac{\text{lbs.}}{\text{g}} \times \frac{\text{ml}}{100 \text{ gallons}}$$

which becomes

$$\frac{\text{g}}{\text{ml}} \times \frac{\text{lbs.}}{453.6 \text{ g}} \times \frac{378,531 \text{ ml}}{100 \text{ gallons}}$$

or

$$\frac{\text{g}}{\text{ml}} \times \frac{834.5 \text{ ml. lbs.}}{(\text{g}) \cdot (100 \text{ gallons})} = \frac{\text{lbs.}}{100 \text{ gallons}}$$

or

$$\text{NaNO}_2 \text{ content (lbs/100 gallons)} = \frac{\text{g NaNO}_2}{\text{ml}} \times \frac{834.5 \text{ ml. lbs.}}{(\text{g}) \cdot (100 \text{ gallons})}$$

If the nitrite is present as KNO_2 , multiply NaNO_2 content (lb/100 gallons) by the ratio: $\frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$

$$\text{KNO}_2 \text{ content (lb/100 gallons)} = (1.23)(\text{NaNO}_2 \text{ content, lb/100 gallons})$$

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

4.005 Determination of Nitrates in Cures and Pickles

In this method, nitrite and nitrate are determined as total nitrate by the Devarda procedure, and the nitrate content calculated by difference. This procedure is not applicable in the presence of nitrogenous organic matter.

4.005A. Theory

When Devarda alloy (50 percent Cu, 45 percent Al, 5 percent Zn) reacts with NaOH, nascent hydrogen is liberated. The action of nascent hydrogen upon nitrites and nitrates will reduce them quantitatively to ammonia. The ammonia is collected in an excess of standard acid solution, and the excess acid titrated with standard alkali solution.

The distillation is performed until the volume of the mixture in the distillation flask is quite low; a relatively high concentration of NaOH being required for complete reduction of nitrites and nitrates to ammonia.

4.005B. Apparatus

- (a) Kjeldahl distillation equipment
- (b) Kjeldahl flask: 800 ml
- (c) Erlenmeyer flask: 500 ml
- (d) Titration apparatus

4.005C. Reagents

- (a) Devarda Alloy
- (b) NaOH solution 50 percent (w/w)
- (c) Standard NaOH solution: 0.2000N (see Protein Determination)
- (e) Indicator solution: Fleisher Methyl Purple
- (f) Carnauba Wax or other suitable anti-foaming preparation

4.005D. Determination

(a) To a 500 ml Erlenmeyer flask, add 25.0 ml of 0.2000N HCl or H_2SO_4 , methyl purple indicator and sufficient distilled H_2O to submerge the end of the delivery tube. Place the flask in position on the distillation assembly.

(b) Weigh 20.0 g of dry cure into a 200 ml volumetric flask.

(1) Add 100 ml distilled H_2O , shake to dissolve cure, dilute to volume with distilled H_2O , stopper and mix well.

(2) Transfer a 20 ml aliquot to an 800 ml Kjeldahl flask.

(c) For pickles, transfer 25.0 ml sample (making no dilutions) to an 800 ml Kjeldahl flask.

(d) Add 275 ml distilled H_2O , 3 g of Devarda alloy, a small quantity of an anti-foaming material and 5 ml of 50 percent NaOH solution, pouring latter down side of flask so that it does not immediately mix with the contents.

(e) Place a glass wool plug in the neck of the flask, connect flask to the distilling bulb on the condenser, rotate flask to mix contents thoroughly, and place flask on heater.

(f) Heat slowly at first, and then at a rate which will yield 250 ml distillate in one hour. Collect 250 ml distillate and titrate the excess standard acid with standard NaOH.

$$\text{Percent total NaNO}_3 = \frac{(V_A - V_B) (8.5) (N)}{S}$$

Where

V_A = Volume of standard acid added

V_B = Volume of standard base used in titration

8.5 = Meq. of NaNO₃ (includes factor of 100 for percentage)

N = Normality of standard acid and base

S = Sample weight in grams represented by aliquot in Kjeldahl flask.

If both the acid and base are 0.2000 N, the equation reduces to:

$$\text{Percent Total NaNO}_3 = \frac{(V_A - V_B) (1.7)}{S}$$

In order to obtain the percent NaNO₃ (due solely to NaNO₃), multiply the sodium nitrite content (percent) (if present) by the ratio NaNO₃/NaNO₂ = 85/69 = 1.23, and subtract this product from the percent total NaNO₃.

Percent NaNO₃ = percent total NaNO₃ - 1.23 (percent NaNO₂)

If the nitrate is present as KNO₃, multiply percent NaNO₃ by the ratio KNO₃/NaNO₃ = 101/85 = 1.19

Percent KNO₃ = (1.19) (percent NaNO₃)

4.005F. Calculations (Pickles)

Because the amount of nitrate which may be added to pickles is measured in pounds nitrate per 100 gallons of pickle, the result should bear the same units.

First express the result in g NaNO₃ per ml of pickle, and proceed from there.

$$\text{Total NaNO}_3 \text{ content (g/ml)} = \frac{(V_A - V_B) (0.085) (N)}{V_P}$$

Where

V_A = Volume of standard acid added

V_B = Volume of standard base used in titration

0.085 = Meq. wt. of NaNO₃

N = Normality of standard acid and base

V_P = Volume of pickle

If both the acid and base are 0.2000 N, the equation becomes:

$$\text{Total NaNO}_3 \text{ content (g/ml)} = \frac{(V_A - V_B) (0.017)}{V_P}$$

In order to convert g/ml to pounds/100 gallons, it is only necessary to multiply the former by 834.5.

In order to obtain the NaNO₃ content, (due solely to NaNO₃), multiply the sodium nitrite (if present) content (lb/100 gallons) by the ratio NaNO₃/NaNO₂ = 85/69 = 1.23, and subtract this product from the total NaNO₃ content.

$$\text{NaNO}_3 \text{ content (lb/100 gallons)} = \text{Total NaNO}_3 \text{ content} - (1.23)(\text{NaNO}_2 \text{ content}).$$

If the nitrate is present as KNO₃, multiply NaNO₃ content by the ratio KNO₃/NaNO₃ = $\frac{101}{85}$ = 1.19

$$\text{KNO}_3 \text{ content (lb/100 gallons)} = (1.19)(\text{NaNO}_3 \text{ content}).$$

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

4.006 Determination of Formaldehyde in Sausage Casings and Patty Paper

4.006A. Theory

A violet color produced by the interaction of formaldehyde and 4, 5-dihydroxy-2,7-naphthalene-disulfonic acid follows Beer's Law. The interfering substances (such as carbohydrate accompanying the casings) are separated by distillation prior to color formation. The color is quite stable and sensitive in the range 1 to 10 micrograms of formaldehyde per milliliter. The method may be applied to other products by following the procedure for Total Formaldehyde.

4.006B. Apparatus

- (a) Condenser.
- (b) Volumetric flasks: 1000 ml and 100 ml.
- (c) Distilling flask: 500 ml.
- (d) Volumetric pipettes: 1 ml.
- (e) Mohr pipette: 10 ml.
- (f) Spectrophotometer suitable for reading at 570 nm.
- (g) Glass beads.
- (h) Test tubes: 30 ml.

4.006C. Reagents

- (a) Phosphoric acid (H_3PO_4) 85 percent ACS.
- (b) Formaldehyde standards.
 - (1) Dilute 5 ml reagent grade HCHO (38 percent) to one liter with distilled water.
 - (2) Dilute 5 ml of (1) to one liter with distilled water. (1 ml = 10 μg).
 - (3) Dilute 50 ml of (2) to 100 ml (1 ml = 5 μg).
- (c) Sulfuric acid solution—Add 300 ml of concentrated sulfuric acid to 150 ml of distilled water. Allow the mixture to cool.
- (d) Chromotropic acid: dissolve 1.13 g of 4, 5-dihydroxy-2, 7-naphthalene-disulfonic acid disodium salt (Practical) or 1.00 g of its acid, in 100 ml of distilled water, filter, and add 400 ml of the above sulfuric acid solution.

4.006D. Sample Preparation

- (a) *Patty Paper* (Free formaldehyde)
 - (1) Soak 10.0 g of patty paper in 200 ml of distilled water for 30 minutes.
 - (2) Remove patty paper and transfer soak water to a 500 ml distilling flask quantitatively using aliquots of distilled water.

(3) Add 2 ml of 85 percent phosphoric acid and a few glass beads.

(4) Distill into a 100 ml volumetric flask and dilute to volume.

(5) Proceed as in Section 4.006E.

(b) *Sausage Casings*

Pre-wash two 10.0 g samples of casing by allowing them to stand in 200 ml distilled water for 30 minutes.

Total Formaldehyde

(1) Transfer a 10.0 g casing sample from the pre-wash to a 500 ml distilling flask containing 150 ml distilled water, 2 ml of 85% phosphoric acid and glass beads.

(2) When the sample is dispersed or broken up, distill into a 100 ml volumetric flask and dilute to volume with distilled water.

(3) Proceed as in section 4.006E.

Free Formaldehyde

(1) Transfer the second 10.0 g casing sample from pre-wash to a 500 ml distilling flask containing 100 ml distilled water.

(2) Stopper, agitate, hold at room temperature 30 minutes, and remove sample.

(3) Add 50 ml distilled water, 2 ml 85 percent phosphoric acid and glass beads to the flask, distill into a 100 ml volumetric flask, and dilute to volume with distilled water.

(4) Proceed as in section 4.006E.

4.006E. Determination

(a) Add 1 ml of the distillate, a distilled water blank and standard solutions to separate clean test tubes.

(b) Add 10 ml chromotropic acid solution rapidly, and mix.

(c) Place tubes in steam or boiling water-bath protected from light, heat 30 minutes, remove, allow to cool, and transfer to cuvettes.

(d) Measure absorbance at 570 nm using the distilled water blank to zero the spectrophotometer. Compare the optical density to a standard curve and calculate parts per million of formaldehyde.

4.006F Standard Curve

(a) Add 0 μ g, 50 μ g, 100 μ g, 200 μ g, 300 μ g, 400 μ g, and 500 μ g, respectively of formaldehyde from standard solution to 100 ml volumetric flasks and dilute to volume with distilled water.

(b) Proceed as in "Determination."

(c) Prepare a curve plotting absorbance versus micrograms of formaldehyde per milliliter; read at 570 nm.

4.006G. Calculations

$$\text{Total or Free Formaldehyde (ppm)} = \frac{AB}{C}$$

A = Micrograms formaldehyde per ml.

B = Final dilution volume in curvette.

C = Sample weight (grams) in final volume.

Combined formaldehyde = Total formaldehyde (ppm) - Free formaldehyde (ppm).

Reference

Official Methods of Analysis of the Association of Analytical Chemists. 10th Edition. 27.031.

4.007 Determination of Animal Fats and Vegetable Fats and Oils

This procedure is applicable to the determination of animal fat as cholesterol in vegetable fats and oils.

4.007A. Theory

A fat or oil is saponified for thirty minutes converting all of the fatty acids to a soap. The aqueous phase is extracted a total of four times with ether and the ether extracts are added to a second separatory funnel. The combined ether extract containing unsaponifiable material is washed with alternate portions of KOH solution and water three times and repeatedly washed with water until the washings are no longer alkaline to phenolphthalein.

The ether extract is evaporated and dried and 1 ml of internal standard solution is added to the unsaponifiable residue. The solution is injected into a gas-liquid chromatograph equipped with a flameionization detector and the cholesterol is quantified using the internal standard procedure.

Controversial samples are given additional cleanup using thin layer chromatography (TLC). The entire sample is spotted and the plate developed, the sterol band is removed and the sterols eluted from the absorbent. The eluant is evaporated, dried, and dissolved in one milliliter of internal standard solution. Gas-liquid chromatography is again used to quantify the amount of cholesterol present. Confirmation is achieved by preparing the acetate derivative of the sterol and using gas-liquid chromatography for a qualitative determination.

4.007B. Apparatus

(a) TLC plates: Prepared from silica gel PF 254 + 366 or HF 254 + 366 (Brinkmann Instruments, Inc., or equivalent) or use precoated plates available as "Uniplate" (precoated with silica gel HF 254 + 366, 500 μ m thick; No. 2112) from Analtech, Inc., 75 Blue Hen Dr. Neward, DE 19711), or "Quanta Gram" (precoated with silica gel PQI-F with 366 nm phosphor, 500 μ m thick; No. 2026) from Quantum Industries, 341 Kaplan Dr. Fiarfield, NJ 07006.

(b) Thin layer plate scraper: Optional-adapt from sealing tube with fritted disk (Corning Glassworks 39580, 30M or equivalent).

(c) Gas chromatograph: Equipped with flame ionization detector and 1 mv strip chart recorder. Temps ($^{\circ}$ C) column, 220-250; detector and flash heater, 240-270; flow rates N_2 (ultra high purity grade), 20-25 psi to elute cholesterol in 8-12 min; H_2 ca 40-45 ml/min; air, 300-340 ml/min. Electrometer sensitivity 1×10^{-9} amp full scale deflection with 1 mv recorder.

Adjust electrometer sensitivity so that 1.5 μ g cholesterol gives ca 50 percent deflection. Repeat injections until constant peak hts are obtained on successive injections of identical volumes of standard mixture.

(d) Preparation of column. Pack glass column, 6' (1.8 m) \times 4 mm id. with commercially available 1-3 percent stationary phase on 100-120 mesh Gas-Chrom Q or dissolve 0.4-1.2 g polysilxane in 200 ml toluene or CH_2Cl_2 -toluene (1 + 1). Heat to dissolve polysiloxane dissolves slowly in solution mixture). (**Caution:** Siloxanes are toxic. Wear disposable gloves and use effective fume removal device when handling.) Add solution to 40 g Gas-Chrom Q and let stand 10 min with occasional gentle stirring. Dry in rotary evaporator held in 50 $^{\circ}$ bath or heat on steam bath with occasional gentle stirring and remove residue solution in vacuum oven at 50 $^{\circ}$ C. (See 4.007C(a))

Carefully wash inside of column and small amount of glass wool with 5 percent solution dichlorodi-methylsilane in toluene, rinse with MeOH until rinsings are neutral to indicator paper, and air dry. Plug column exit with small plug of silanized glass wool. Add coated packing material through injection port, using funnel and plastic tubing and tapping column very gently during addition. Add one-fourth packing material at a time, remove funnel, and apply ca 5-10 psi N_2 to injection port while tapping gently to settle packing. Pack to 2.5 cm from injection port and plug with silanized glass wool.

(1) Conditioning of column. Heat 8 hr at 260 $^{\circ}$ C with ca 5-10 psi N_2 flowing through column. Shut off pressure, raise temp. to 290 $^{\circ}$ C and continue heating \geq 8 hours. Reduce temp. to 260 $^{\circ}$ C, adjust N to 5-10 psi, and heat additional 12-24 hours.

(2) Performance. Chromatograph ca 2 μ l cholesterol- β -sitosterol standard mixture to determine retention times and resolution of column. Min. of 1600 theoretical plates is required for cholesterol peak; theoretical plates = $(L/B)^2 \times 16$, where L = cm cholesterol peak from injection point, and B = cm triangulated base width of cholesterol peak. In addition, separation of cholesterol and campesterol peaks, expressed as peak resolution, should be 2.2. Peak resolution = $2D/(B + P)$, where D = distance in cm between cholesterol and campesterol peak maximum, B = triangulated base width of cholesterol peak; and P = triangulated base width of campesterol peak. Determine peak resolution on sample having ca equal amounts cholesterol and campesterol (ca equal peak areas); sample injected should give peak hts 25-50 percent of FSD.

(e) Separatory funnel; 125 ml.

(f) 4 dram vials with aluminum lined caps.

4.007C. Reagents

(a) GLC column packing: (1) Stationary phase. JXR, or OV-1, or OV-101 dimethyopolysiloxane, or OV-17, or OV-22 methylphenylpolysiloxane. (2) Support. 100-120 mesh Gas-Chrom Q. Commercially prepared packing of 1 or 3 percent stationary phase available from Applied Science Laboratories, Inc., or Supelco, Inc.

(b) Ethyl acetate (EtOAc): Distilled in glass or equivalent.

(c) Cholestane standard soln: 0.4 μ g/ μ l. Weigh 40.0 mg cholestane standard (Applied Science Laboratories, Inc.) into 100 ml vol. flask and dilute to volume with EtOAc.

(d) Cholestane internal standard soln: 0.2 μ g/ μ l. Dil. 10.0 ml std soln, (c), to 20.0 ml with EtOAc.

(e) Chloroform: Distilled in glass or equivalent.

(f) Ethyl ether: Anhydrous 0.01 percent alcohol.

(g) Petroleum ether: Distilled in glass, bp 30-60°C or equivalent.

(h) β -Sitosterol standard soln: 3 μ g/ μ l. Weigh 30.0 mg β -sitosterol std (Aldrich Chemical Co., Inc.) into 10 ml volumetric flask and dilute to volume with EtOAc. Commercial material is mixture of campesterol (earlier eluting component) and B-sitosterol.

(i) Cholesterol standard soln: 1.2 μ g/ μ l. Weigh 60.0 mg cholesterol std (Applied Science Laboratories, Inc.) into 50 ml volumetric flask and dilute to volume with EtOAc.

(j) Cholestane-cholesterol standard mixture: 0.2 μ g cholestane and 0.6 μ g cholesterol/ μ l. Mix equal volumes cholestane (c), and cholesterol standard solutions.

(k) Cholesterol- β -sitosterol standard mixture: 0.6 μ g cholesterol and 1.5 μ g β -sitosterol/ μ l. Mix equal volumes cholesterol and β -sitosterol, (h) standard solutions.

(l) Cholesteryl acetate standard solution 0.5 μ g/ μ l: Weigh 30.0 mg cholesteryl acetate std (ICN Pharmaceuticals, Inc., Life Sciences Group) into 50 ml volumetric flask and dilute to volume with EtOAc.

(m) KOH solution (3 + 2).

(n) Ethyl alcohol: Reagent grade.

(o) Pyridine: Reagent grade.

(p) Acetic anhydride: Reagent grade.

Note: For qualitative determination, inject $10\mu\text{l}$ of solution from 4.008 D.(p), below, and compare with $4\mu\text{l}$ injection of standard cholesterol, 4.008 C(i). If this qualitative test indicates the presence of animal fats in vegetable oil, evaporate the entire remaining solution to dryness under N_2 , store in freezer. Proceed as in step 4.008 E.(a).

4.007D. Sample Preparation

(a) Accurately weigh 2-2.5 g fat into saponification flask (200 ml Erlenmeyer with standard taper 24/40 outer joint is recommended).

(b) Add 25 ml alcohol and 1.5 ml (3 + 2) KOH soln. Saponify by boiling, with occasional swirling, on steam bath 30 min under reflux air condenser. (No loss of alcohol should occur during saponification).

(c) Transfer alcohol-soap solution while still warm to 250 ml separator, using total of 50 ml H_2O . Rinse saponification flask with 50 ml ether and add ether to separator, using total of 50 ml H_2O . Rinse saponification flask with 50 ml ether and add ether to separator.

(d) Shake vigorously, and let layers separate and clarify.

(e) Drain lower layer back into saponification flask and pour ether layer through top into second separator containing 20 ml H_2O . Rinse pouring edge with ether, adding rinsings to second separator.

(f) Extract soap solution in the saponification flask with two 50 ml portions ether in same manner.

(g) Make total of 4 extractions for marine oils or other oils of high unsaponifiable content.

(h) Rotate combined ether extracts gently with the 20 ml H_2O (violent shaking at this stage may cause troublesome emulsions).

(i) Let layers separate and drain aqueous layer.

(j) Wash with two additional 20 ml portions H_2O , shaking vigorously.

(k) Then wash ether solution 3 times with alternate 20 ml portions ca 0.5N aq KOH and H_2O , shaking vigorously each time.

(l) If emulsion forms during washing, drain as much aqueous layer as possible, leaving emulsion in separator with ether layer, and proceed with next washing.

(m) After third KOH treatment, wash ether solution successively with 20 ml portions H_2O until washings are no longer alkaline to phenolphthalein.

(n) Quantitatively transfer the ether extract to a 250 ml beaker and evaporate to dryness under nitrogen.

(o) Dissolve unsaponifiable matter in 4-5 ml CHCl_3 , and mix on vortex mixer for about 30 seconds.

(p) Transfer to 25 ml volumetric flask, repeat with four more aliquots of CHCl_3 , adjust to mark, and store in freezer.

4.007E. Isolation of Sterols by Thin Layer Chromatography

(a) preparation of Plates

(1) Align 5 matching 20×20 cm glass plates on mounting board, and just before coating, wipe plates with tissue dampened with alcohol to remove any dust or fingerprints.

(2) Adjust applicator to deliver 0.5 mm thick layer.

(3) Weigh 45 g silica gel into 500 ml Erlenmeyer, add 130 ml H₂O, shake vigorously 25-30 sec, and pour into applicator.

(4) Immediately coat plates with silica gel suspension and let plates rest undisturbed until gelled (0.5-1 hr).

(5) Dry coated plates 2 hr at 110°C and store in desiccating cabinet until just before use.

(b) Thin Layer Chromatography.

Line developing chamber with blotting paper and add 100 ml ether-pet ether (1 + 1) to chamber. Cover chamber and equilibrate 2 hr. Draw line across plate 17 cm from bottom and ca 1 cm from each side. Spot 10 μ l μ β -sitosterol standard solution (c), at point 2 cm from bottom edge and 3 cm from one side of plate. Dissolve unsaponifiable matter in 200 μ l CHCl₃ and spot entire sample in 10 μ l portions on imaginary line 2 cm from bottom edge of plate so that spot centers are 0.75 cm apart. Rinse vial with ca 100 μ l CHCl₃ and spot rinse solution in equal portions on top of sample spots. Immediately insert plate into equilibrated chamber (position plate to expose coated surface to max chamber volume); cover chamber and seal with tape. Withdraw plate from chamber when solvent front reaches 17 cm stop line. Evaporate solvent and view plate under long-wave UV light in darkened room. Mark off sterol band (same R_f, 0.2-0.3, as β -sitosterol standard) with needle, and remove sterol band as follows (do not remove β -sitosterol standard): Scrape off sterol band with square end of stainless steel spatula into 100 ml beaker and transfer with 20 ml CHCl₃ to 70 mm top diameter funnel containing folded 12.5 cm diameter filter paper. Extract sterols with five 10 ml portions CHCl₃ and evaporate combined filtrate to near dryness on steam bath under nitrogen. Transfer residue to 4 dram vial (screw-cap with Al liner) with CHCl₃ and evaporate to dryness under nitrogen. (Alternatively, remove sterol band with TLC plate scraper, elute sterols from silica gel with 70 ml CHCl₃ (fourteen 5 ml portions), and evaporate solvent to near dryness on steam bath under nitrogen).

4.007F. Gas Chromotography of Sterols

(a) Pipet 1.0 ml cholestane internal std soln into 4 dram vial contg extd sterols, rotate vial to wash down sides with internal std soln, and swirl to dissolve sterols.

(b) Inject 2 μ l sample at least in duplicate. Repeat with 2 μ l cholestane-cholesterol peak ht in sample is 60 percent full scale deflection, add addnl 1.0 ml cholestane internal standard soln to sample and chromatograph sample and standard mixt as above.

Measure cholestane and cholesterol peak hts in mm. Calculate mg cholesterol/100 g sample, correcting for internal std, as follows:

$$(1) \text{ mg cholesterol/100 g} = (H_1/H_s) \times (C_s/C_1) \times (S_s/S_1) \times (Q_1/Q) \times 100$$

H₁ and H_s = ht (mm) cholestane and cholesterol peaks respectively in standard mixture.

S_s and S₁ = ht (mm) cholesterol and cholestane peaks respectively in sample.

C_s and C₁ = μ g cholesterol and cholestane/ μ l, respectively in the standard mixture.

Q₁ = μ g cholestane/ μ l in sample

Q = mg sample/ μ l.

$$(2) \text{ Percent Animal fat} = \frac{(\text{mg cholesterol/100 g} - 1.5 \text{ mg/100 g})}{82.0 \text{ mg/100 g}} \times 100$$

1.5 mg/100* g = cholesterol-like component due to edible vegetable oils.

82.0 mg/100 g = the average cholesterol content of beef and pork fat.

4.007G. Confirmatory Test

Presence of cholesterol may be confirmed by GLC of sterol acetates. After determining cholesterol by GLC, evaporate sample to dryness on steam bath under nitrogen. Cool and add 3 ml pyridine and 1 ml acetic anhydride. Cap vial, swirl on steam bath until sterols dissolve, and continue heating on steam bath 1 hr. Evaporate using nitrogen stream, until no odor of pyridine is detected. Chromatograph sterol acetates and cholesteryl acetate standard solutions and compare retention times of sample and cholesteryl acetate peaks.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

*Some vegetable oils, notably palm oil, or those containing mono and diglycerides derived from animal fat, may have higher levels of this component present. In that case a sample of the oil, prior to processing, will be required to determine the amount of the component (mg/100g).

4.008 Determination of Antioxidants in Rendered Fat from Processed Products, Lard, Shortenings, and Oils

4.008A. Theory

The prevention of oxidative degradation in fats may be controlled by the use of synthetic phenolic antioxidants. Specific acceptable antioxidants in the U.S., and their respective limits in meat are found in the Regulations. This test detects the presence in fats, oils, and shortenings, and quantitates the amount of, 2- and 3-tert-butyl-4-hydroxyanisole (BHA); tert-butyl-hydroquinone (TBHQ); 3,5-di-tert-butyl-4-hydroxytoluene (BHT); 2,6-di-tert-butyl-4-hydroxymethylphenol (Ionox-100); 2,4,5-trihydroxybutyrophenone (THBP); propyl gallate (PG); octyl gallate (OG); dodecyl gallate (DG); and nordihydroguaiaretic acid (NDGA). The antioxidants are extracted from fat with hexane saturated with acetonitrile and partitioned into acetonitrile saturated with hexane, concentrated under vacuum, and determined by reverse phase gradient elution high performance liquid chromatography (HPLC) with detection at 280 nm. The mobile phase is water-acetonitrile with 5 percent acetic acid. With this system, only Ionox 100 and OG are not resolved. Another mobile phase is used to resolve these two antioxidants.

4.008B. Reagents

(a) Acetonitrile — Distilled-in-Glass grade, or equivalent.

(b) 2-propanol — Distilled-in-Glass grade, or equivalent.

(c) Hexane — Distilled-in-Glass grade, or equivalent.

(d) Methanol — Distilled-in-Glass grade, or equivalent.

(e) Acetic Acid — A.R. grade.

(f) Mobile Phases

(1) Distilled water — Millipore or HPLC grade bottled. Add 5% v/v A.R. grade acetic acid.

(2) Acetonitrile — Use spectrograde or quality suitable for HPLC. Add 5 percent % v/v A.R. grade acetic

(3) Methanol — Use spectrograde or quality suitable for HPLC. Add 5 percent, % v/v A.R. acetic acid.

(g) Antioxidants — BHA, BHT, TBHQ, Ionox 100, THBP, and PG (Available from Polyscience Corporation, Niles, Illinois, 60648); OG and DG (Available from Naarden, The Netherlands); NDGA (Food Chemicals Codex Reference Standard); or equivalents.

(h) Standard Solutions — Refrigerate all antioxidant solutions out of direct light. Prepare all solutions with 2-propanol-acetonitrile (1 + 1).

(1) Stock Solution I — 1 mg/ml. Accurately weight and transfer 50 mg each of PG, TBHQ, NDGA, OG, and BHT into one 50 ml volumetric flask, dissolve, dilute to volume, and mix.

(2) Stock Solution II — 1 mg/ml. Accurately weigh and transfer 50 mg each of THBP, BHA, Ionox 100, and DG into one 50 ml volumetric flask, dissolve, dilute to volume, and mix.

(3) Standard Solution I — 0.01 mg/ml (10 µg/ml). Pipet one ml stock solution I into 100 ml volumetric flask, dilute to volume, and mix.

(4) Standard Solution II — 0.01 mg/ml (10 µg/ml). Pipet one ml stock solution II into 100 ml volumetric flask, dilute to volume, and mix.

(5) Standard Solution I & II — 0.01 mg/ml (10 µg/ml). Pipet one ml each of stock solutions I and II into 100 ml volumetric flask, dilute to volume, and mix.

(i) Extracting Solvent — Saturate hexane with acetonitrile by shaking together for 2 minutes in a separatory funnel. Allow to separate and use the hexane layer for extracting antioxidants from fat.

(j) Partitioning solvent — Use acetonitrile layer from 4.008B(i), above.

(k) Anhydrous Sodium Sulfate, reagent grade

4.008C. Apparatus

(a) Liquid Chromatograph — Waters Model No. 244, equipped with Model No. 660 solvent programmer operated at ambient conditions at 2 ml per minute with Model No. U6K sample injector valve, or equivalent. A suitable uv detector with sensitivity range 0.01-2 absorbance units full scale (AUFS), measuring absorbancies at 280 nm.

(b) Chromatographic Columns

(1) Analytical Column — 4.6 × 250µm id stainless steel column packed with 10 µm Li Chrosorb RP-18 (Altex Scientific Inc.), or equivalent.

(2) Stainless steel guard column — 42 × 2.6 mm id, packed with 38 µm mean diameter LiChrosorb RP-2 (E Merck, Darmstadt, (GFR), or equivalent, placed in line just before the analytical column, using zero dead volume fittings.

(c) Food chopper, suitable for grinding processed products.

(d) Steam bath

(e) 125 ml and 250 ml separatory funnels.

(f) 250 ml round bottom flasks.

(g) Rotary Flask Evaporator — Rinco, or equivalent, with ≤ 40°C. water bath.

(h) Disposable pipets.

(i) 10 ml stoppered graduated cylinders.

(j) Filter Paper — coarse to fit into apparatus (k).

(k) Powder Funnels — to fit into 250 ml beakers.

(l) 50 ml, 150 ml, and 250 ml beakers.

(m) Balance top loader, (Mettler P1200 or equivalent).

(n) 50 ml and 100 ml volumetric flasks.

(o) 25 ml pipets.

(p) 1 ml pipets.

4.008D. Determination

(a) Processed Products and Fats — Pass sample through food chopper with suitable quantity of anhydrous sodium sulfate to combine with moisture in sample. Do not use sodium sulfate if sample is fat only. Place chopped sample (Sufficient to yield 10-25 g fat) in a beaker. Slurry with hexane saturated with acetonitrile and filter through coarse filter paper into 250 ml beaker. Evaporate solvent on steam bath go to step (c).

(b) Liquid oils — Accurately weigh 20.0 g oil into 50 ml beaker and quantitatively transfer to 100 ml vol flask, rinsing beaker with hexane, saturated with acetonitrile. Dilute to volume with hexane saturated with acetonitrile and mix. Go to step (e).

(c) Accurately weigh 10 g lard, shortening, or extracted fat into 150 ml beaker. Add ca 30 ml hexane saturated with acetonitrile and dissolve sample, heating gently if necessary.

(d) Quantitatively transfer dissolved fat to 100 ml volumetric flask, rinsing beaker with hexane saturated with acetonitrile; dilute to volume, and mix.

(e) Pipet 25 ml aliquot into 125 ml separatory funnel and partition with 3-50 ml portions of acetonitrile saturated with hexane. If emulsions form, break by holding separatory funnel under hot tap water for 5-10 seconds.

(f) Collect partitions in 250 ml separatory funnel and let combined partitions slowly drain into 250 ml round bottom flask to aid removal of hexane/fat droplets.

(g) Evaporate to 3-4 ml using rotary evaporator with $\leq 40^{\circ}\text{C}$, water bath.

NOTE: Evaporation should be accomplished in <10 min to avoid loss of TBHQ by oxidation. A good water aspirator is mandatory.

(h) Using disposable pipet, transfer acetonitrile/fat droplet mixture to 10 ml stoppered graduated cylinder. Rinse flask and disposable pipet with small portions of non-hexane saturated acetonitrile, transferring rinsings to graduated cylinder with the pipet, until 5 ml are collected. Rinse flask and disposable pipet with small portions of 2-propanol, transferring all rinsings to graduated cylinder until exactly 10 ml is collected. Mix contents of the cylinder.

(i) Using sample injector, inject in duplicate, 20 μl from graduated cylinder into instrument with column as described in apparatus (a) and (b), above. Set range to 0.05 or 0.1 AUFS. For sample peaks off scale, accurately dilute sample solutions with 2-propanol-acetonitrile (1 + 1). Inject appropriate amount of standard solution 4.008B, h (3), and/or (4).

Use linear gradient, programmed from 30% reagent (f)2 in (f)1 to 100%(f)2, over 10 minutes with 4 minutes hold at 100%(f)2 ml/min. Increase flow rate to 4 ml/min, at 100%(f)2 for 5 minutes to elute nonpolar lipids. Return to 30%(f)2 over 1 minute at 1 ml/min, and let baseline, pressure, and solvent composition stabilize (ca 10 min.). Back pressure of ca 1500 psi is attained at 2 ml/min, at 30% acetonitrile in water and 2300 psi at 4 ml/min, at 100% acetonitrile.

If presence of Ionox-100 or OG is indicated, identify and quantitate using reagents (f)1 and (f)3 with linear gradient of 35% (f)1 in (f)3 to 100% (f) over 10 minutes with 4 minutes hold at 100% (f)3 at 1 ml/min. Increase flow rate to 4 ml/min, at 100% (f)3 for 5 minutes. Return to 35% (f)1 in (f)3 over 1 minute at 2 ml/min, and let baseline, pressure, and solvent composition stabilize (ca 10 min.). (Note: If both ionox and OG are present, accurate quantitation may not be possible.)

4.008E. Identification and Quantification

(a) Identify peaks as described in Fig. 1 and Table I, below. Each analytical system may vary slightly:



Fig. 1 — Chromatographic separation of antioxidant standards, ca 80 ng each: 1, PG; 2, THBP; 3, TBHQ; 4, NDGA; 5, BHA; 6, Ionox-100; 7, OG; 8, DG; 9, BHT.

Upper chromatogram, reagent (f)1, 5% (v/v) acetic acid in water, reagent (f)3, 5% (v/v) acetic acid in methanol with linear gradient of 35% (f)1 in (f)3 to 100% (f)3 over 10 min.; lower chromatogram, reagent (f)1, 5% (v/v) acetic acid in water, reagent (f)2, 5% (v/v) acetic acid in acetonitrile with linear gradient of 30% (f)2 in (f)1 to 100% (f)2 over 10 min.

Table 1 Retention times and Detector Response for Antioxidants

Antioxidant	Retention time, min. ³		% FSD for 80 ng ^b
	Water-Acetonitrile	Water-Methanol	
PG	5.1	7.4	41
THBP	6.3	8.6	56
TBHQ	7.1	8.9	13
NDGA	8.1	10.6	25
BHA	9.6	11.2	15
Ionox-100	10.0	11.7	9
OG	10.0	11.9	49
DG	12.7	13.9	39
BHT	13.1	13.9	11

^a Retention time of unretained compound (NaNO₃) is 1.4 min.

^b Percent full scale deflection (FSD) at 0.05 absorbance unit full scale with 5% acetic acid cetonitrile-water gradient.

(b) Calculation

Determine average peak height or average peak area of antioxidant containing sample from duplicate injections, average peak height or average peak area of antioxidant standard from duplicate injections, and calculate concentration of antioxidant as follows:

$$\text{Antioxidant, ppm} = (C_s/R_s) \times (R_x/W_x) \times D$$

$$\% \text{ Antioxidant in fat} = (\text{ppm}) 10^{-1}$$

where:

R_x = average sample peak height or average sample peak area

R_s = average standard peak height or verage standard peak area

C_s = concentration of standard in ug/ml

W_x = weight of sample in g/ml in 10 ml final extract

D = dilution factor if solution injected is diluted. R_x and R_s must be in the same units, i.e., height or area.

Reference:

Page, B. Denis, J. Assoc. Off. Anal. Chem. (Vol. 62, No. 6, 1979), (Vol. 66, No. 3, 1983)

4.009 Determination of Na^+ , K^+ , and Cl^- by Ion Specific Electrode Using the NOVA Analyzer

4.009A. Theory

The NOVA analyzer uses ion specific electrode technology to measure the activity of Na^+ , K^+ , and Cl^- in aqueous solution. The instrument originally designed to measure blood electrolyte levels in whole blood has been modified to measure Na^+ , K^+ , and Cl^- in aqueous extracts of food products. The instrument is highly automated and self contained. Calibration fluids and sample dilution buffers are contained in a fluids pack within the cabinet. Operation is simple, and routine maintenance may be performed by a properly trained operator.

4.009B. Apparatus

(a) NOVA Analyzer: NOVA Biomedical Corp., 20 Ossipee Rd., Newton, MA 02164, equipped with Na^+ , K^+ , and Cl^- electrodes, auto sample unit and printer.

(b) Macropipette 5000 ul: Supelco #5-8471, or equivalent.

(c) Sample cups: NOVA Biomedical Corp., 20 Ossipee Rd., Newton, MA 02164, or equivalent.

(d) Centrifuge tubes: disposable polypropylene 50 ml capacity with screw caps (Corning #25330, or equivalent).

(e) Disposable macropipette tips: Supelco #5-8461, or equivalent.

(f) Disposable macropipette filter tips (45 micron): Supelco #5-8474, or equivalent.

(g) Eberbach mechanical shaker, or equivalent.

4.009C. Reagents

(a) Deionized water: good quality deionized water with resistivity greater than 10 megohms.

(b) Fluid Pack: NOVA Biomedical Corp., 20 Ossipee Rd., Newton, MA 02164.

(1) High Standard: Na^+ (230 ppm), K^+ (390 ppm), Cl^- (709 ppm).

(2) Low Standard: Na^+ (23 ppm), K^+ (39 ppm), Cl^- (70.9 ppm).

(3) Sample dilution buffer.

(4) Reference electrode solution.

(c) Electrode cleaning solution.

(d) Standard Solutions.

(e) Sodium Conditioning Solution.

(1) Stock Solutions.

(a) 100 ug/ml Na^+ : Dry reagent grade NaCl 2 hr. at 100°C, cool and weigh 2.5421 g into a 1-liter volumetric flask. Dilute to volume with deionized water.

(b) 1000 ug/ml K^+ : Dry reagent grade KCl 2 hr. at 100°C, cool and weigh 1.9068 g into a 1-liter volumetric flask. Dilute to volume with deionized water.

(2) Working Solutions

(a) Prepare working standard solutions by making the following dilutions from the 100 ug/ml stock solutions using deionized water.

Vol Na ⁺ Stock	Vol K ⁺ Stock	Final Volume	(PPM)		
			Na ⁺	K ⁺	Cl ⁻
10 ml	7.5 ml	200 ml	50	37.5	111
20 ml	15 ml	200 ml	100	75.0	222
40 ml	30 ml	200 ml	200	150	444
80 ml	60 ml	200 ml	400	300	888

(3) Recovery spiking solution

Dry reagent grade NaCl and KCl 2 hrs. at 100°C. Cool and weigh 7.6203 g NaCl and 1.7161 g KCl into a 500 ml volumetric flask. Make to volume with deionized water.

4.009D. Sample Preparation and Storage

Solid or semi-solid sample materials should be homogenized as per instructions contained in Section 1.002B III. Fluid or semi-fluid sample materials such as soups, stews, etc., should be blended. A standard commercial style food processor has been found to be adequate for this purpose. Waring Blenders or Virtis homogenizers are also acceptable. Sub samples of homogenized material could be stored frozen until analyzed. Plastic bags are recommended for storage.

4.009E. Determination

(a) Instrument Calibration — Calibrate the NOVA instrument according to manufacturers instructions. Internal electrode calibration is performed automatically using standard reference solutions contained within the fluid pack. Do not proceed until a satisfactory calibration is obtained.

(b) Standard Curve — Analyze the four working standards prepared in 4.013C(e)2. Calculate a calibration curve of the form $y = Mx + B$ for each ion where y = actual ppm present, x is ppm determined by NOVA, M is the slope and B the intercept. M and B may be calculated using the following formulas:

$$B = \frac{\sum_{xy} - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \quad M = \bar{y} - B\bar{x}$$

where: $\bar{y} = \frac{\sum y}{n}$; $\bar{x} = \frac{\sum x}{n}$

$$\text{Regression Coefficient} = \sqrt{\frac{\left[\sum_{xy} - \frac{\sum x \sum y}{n} \right]^2}{\left[\sum x^2 - \frac{(\sum x)^2}{n} \right] \left[\sum y^2 - \frac{(\sum y)^2}{n} \right]}}$$

Do not proceed with sample analysis until satisfactory linear regression data are obtained.

Each set of samples should contain the following quality assurance samples:

(1) Internal Check — a sample which has been analyzed previously and which has a known Na^+ , K^+ and Cl^- content.

(2) Recovery Blank — a sample to which no Na^+ , K^+ , and Cl^- has been added.

(3) Recovery — a sample to which a known amount of Na^+ , K^+ , or Cl^- has been added. Prepare by adding 2.0 ml of recovery spiking solution to recovery blank from above (equivalent to 12,000 ug Na^+ , 3,600 ug K^+ , and 21,768 ug Cl^- added).

(c) Sample Extraction:

(1) Weigh 2.5 g of homogenized sample into a 50 ml polypropylene centrifuge tube.

(2) Quantitatively add 40 ml \pm 0.1 ml deionized water to each sample.

(3) Cap and place horizontally on shaker.

(4) Shake for 30 minutes.

(5) Let stand for 15 minutes.

(6) Transfer about 2 ml of the supernatant fluid to a sample cup using a 45 micron filter tip assembly.

(7) Analyze on NOVA analyzer as per instrument instructions.

4.009F. Calculations

Data from the NOVA analyzer are reported in ppm on a solution basis and therefore must be converted to mg/100 g of product. The following steps are required to perform this conversion:

(a) Raw data from the NOVA analyzer are corrected using the linear curves calculated in Section 4.013E, above.

(b) Mg/100 g sample is calculated using the following formula:

$$\text{Mg/100g} = \frac{R \times V}{W \times 10}$$

Where: R = Recovery corrected ppm NOVA reading

V = Volume of extract (usually 100 ml)

W = Sample weight in grams

10 = Conversion factor

Calculations of linear regression coefficients and sample concentrations are easily accomplished using a suitably programmed calculator or computer.

4.009G. Quality Control Criteria

In order to ensure the reliability of reported results the following criteria are monitored:

(a) Sample identity is carefully preserved throughout the process. Working conditions are arranged to reduce the possibility of loss of sample identity.

(b) Reagents and solutions are carefully prepared and recorded. Calculation of reagent or reference standard concentrations are checked by a second analyst.

(c) Instrument performance is carefully monitored. Before sample analysis the instrument must pass the following tests:

(1) Internal calibration — the instrument must calibrate successfully with an acceptable slope for all three electrodes.

(2) Linear regression curves calculated using working standards must have acceptable slopes and regression coefficients.

(3) The value for the internal check sample must be within ± 2 standard deviation of the mean value previously determined on a minimum of 14 checks.

(4) The recovery values must be within acceptable limits. (See Sec. 4.013H. Appendix II for expected recovery values).

(5) As a final check for each sample the ratio of Na^+ to NaCl is calculated. This ratio should be reasonably close to the 0.39 theoretical value.

4.009H. Appendix

Recovery Values

Added	Mean Recovery	Std Dev.	CV	n
Na^+	100.5	2.65	2.64	14
K^+	100.7	2.44	2.42	14
Cl^-	100.3	4.22	4.21	14

4.010 Determination of Sodium and Potassium in Meat by Atomic Emission Spectroscopy

4.010A. Theory

Due to expected high levels of Na and K in products of interest, atomic emission rather than atomic absorption is used. The sample is defatted and ashed. Wet ashing is the primary ashing technique, but dry ashing may be necessary for some products. The resultant ash is solubilized, diluted, aspirated into a flame, its emission read and compared to a standard curve for quantitation.

4.010B. Apparatus

- (a) Atomic emission spectrophotometer (P/E Model 603, or equivalent).
- (b) Food Chopper equipped with $\frac{5}{64}$ inch perforated front plate.
- (c) Food processor with rotary blades.
- (d) Steam bath.
- (e) Explosion proof hot plate.
- (f) Convection oven.
- (g) Muffle Furnace.
- (h) Platinum crucibles.
- (i) Centrifuge
- (j) Vinyl gloves
- (k) Desiccator
- (l) Analytical balance

(m) Glassware: 50 ml Pyrex beakers, class A volumetric flasks and transfer pipets and 50 ml plastic Falcon tubes with lids. All glassware must be cleaned with 50 percent HNO_3 and rinsed with H_2O immediately before use.

4.010C. Reagents

- (a) Distilled water — free from Na and K; either doubled-distilled or deionized. *Use for all " H_2O " references in this method.*
- (b) Petroleum ether — distilled in glass (Burdick and Jackson, or equivalent).
- (c) Nitric Acid — redistilled. *Use for all " HNO_3 " references in this method.*
- (d) Sodium standard solutions:
 - (1) Na stock solution, 1mg Na/ml. Dry reagent grade NaCl 2 hr at 110°C ; cool in desiccator. Weigh 2.5421g into 1 liter vol flask and dilute to vol with H_2O .

(2) Na working solutions (for flame emissions) — 20, 40, 80, 150, 300, and 400 ug Na/ml. Pipet 2, 4, 8, 15, 30, and 40 ml Na stock solution into separate 100 ml vol flasks; add 2 ml HNO_3 and 10 ml K stock solution to each flask; dilute to volume with H_2O . Use a solution of 2 ml HNO_3 diluted to 100 ml with H_2O to zero spectrophotometer. Make a new set of working solutions for each set of samples analyzed.

(e) Potassium standard solutions:

(1) K Stock solution — 1 ml K/ml. Dry reagent grade KCL 2 hr at 100°C; cool in desiccator. Weigh 1.9068 g into 1 liter vol flask dilute to vol with H₂O.

(2) K working solutions (for flame emission) — 20, 40, 60, 100, 150, and 180 ug K/ml. Pipet 2, 4, 6, 10, 15, and 18 ml K stock solution into separate 100 ml vol flask; add 2 ml HNO₃ and 30 ml Na stock solution to each flask; dilute to volume with H₂O. Use solution of 2 ml HNO₃ diluted to 100 ml with H₂O to zero spectrophotometer. Make a new set of working solutions for each set of samples analyzed.

4.010D. Determination

(a) Sample Preparation — (See "Chemistry Laboratory Guidebook" Sec. 1.002) — Convert the sample to a homogenous mass containing all the original juices free liquids, chunks, and add materials so that the small portion taken for analysis is representative of the sample.

(1) Commminuted meat food products — Chop twice using a clean, dry chopper equipped with a plate perforated by $\frac{3}{64}$ inch holes, with mixing between passes through the chopper. Maintain sample identification.

(2) Meat cuts — Chop three times as described in 4.014 D.(a)(1), above.

(3) Pizza, spaghetti products, meat and poultry pies, soups, and stews — Homogenize, using a food processor with rotating blades and weigh immediately into a clean 50 ml beaker before settling begins.

(b) Ashing

(1) Wet Ashing

(i) Weight 1.500 to 2.500 g to the nearest 0.001 g into a 50 ml Pyrex beaker. Flatten samples to cover at least one-half of the bottom of the beaker before final weight is taken.

Keep sample in a flat wafer (as much as possible) rather than small pieces. This will prevent loss of sample during defatting. Dry 16 hr at 90°C and cool.

(ii) Samples with unknown or known high oil/fat content — Add about 10 ml pet ether and warm on steam bath or an explosion proof hot plate, set at low heat, until oil/fat is extracting. Decant and repeat until sample is defatted. Evaporate remaining pet ether. Proceed as in next paragraph beginning with, "add 5ml"

(iii) Samples with low oil content — Add 5 ml HNO₃ to each beaker. Digest on low temp hot plate (200-250°F) until sample dissolves. Samples containing products other than meat, such as pizza and spaghetti, react faster and must be started below 200°F. Evaporate to dryness. Repeat this digestion and evaporation two more times with 5 ml HNO₃. (Stop at this point. Do not continue unless samples can be carried through to completion or losses will occur.) Add 2 ml HNO₃ and warm to dissolve. Proceed as in next paragraph, beginning with, "Transfer digest"

(iv) For flame emission, transfer digest to 100 ml vol flask with hot H₂O; wash down sides of beaker three times with hot H₂O, add washings to the flask. Cool and dilute to vol with H₂O. Centrifuge 30 ml aliquot in 50 ml Falcon tubes at 2000 rpm for 10 min.

(v) Prepare two blank solutions by carrying two empty 50 ml beakers or crucibles through the method as described.

The average of the blank Na/K emission units are subtracted from the Na/K analytical sample emission units before calculating Na/K content of sample.

(vi) Analyze a recovery with each set by fortifying samples of known Na/K content with approximately 600 mg Na/100 mg and 180 mg K/100 mg.

(2) Dry Ashing (For samples that are difficult to wet ash)

(i) Prepare sample as in 4.014D.a) weigh appropriate amount of samples into a platinum crucible and dry at 90°C for 16 hr. Place in a cold muffle furnace and slowly bring up to 525°C. Ash until little or no visible carbon is present. Cool, and dampen ash with 2 ml 50 percent HNO₃. Dry on hot plate set at 200°F. Return to cool muffle furnace and bring to 525°C. Hold for 1 hr; Cool. (Stop at this point if samples can not be carried through to completion). Dissolve ash with dilute HNO₃ (20 ml HNO₃ per liter of H₂O). Transfer quantitatively to a 100 ml vol flask and make to volume with dilute HNO₃.

(ii) Same as 4.014 D (b)(1)(v), above.

(iii) Same as 4.014 D (b)(1)(vi), above.

(c) Emission Spectrophotometry

(1) Follow manufacturer's directions for type of instrument available. Read at 589 nm for Na (for the P/E 603: 295 vis-EM chop, slit 4, 4 sec) and a 767 nm for K (for the P/E 603: 383 vis-EM chop, slit 4, 4 sec). Warm burner 20 minutes to stabilize flame.

(2) Read standard curve: the curve can be read several ways. If the entire curve is to be used (Na: 20 to 400 ug Na/ml, K: 20 to 180 ug K/ml), keep the highest standard used between 1.6 and 1.7 emission units. Six standards evenly spaced along the curve are normally used. If only a short portion of the curve is to be used, four standards may be used. Even if a short portion is used, set the 180 ug K/ml and 400 ug Na/ml standards at 1.6 to 1.7 emission units. The sample readings must be between the highest and lowest standard used. Check a standard every 5 to 10 samples and watch for zero drift. Treat the blank solutions and the recoveries the same as the samples. If any samples require further dilution, read the blanks diluted to the same volume as the samples.

Note: Contamination can be a problem. Sodium is present everywhere. It is recommended that analysts wear vinyl gloves, not only to avoid contamination but also to protect their hands from HNO₃. Paper towels, filter papers, detergent, tapwater, water in steam baths, most distilled water and glass wool all contain significant amounts of Na and must be avoided. The redistilled H₂O used to flush the burner system every 5 to 10 samples (just prior to checking a standard) should be changed frequently. Significant drifting of zero can occur if the burner head gets dirty. Air drifts can cause the signal to be noisy due to temperature changes in the flame. Protect burner from drafts if this becomes a problem.

4.010E. Calculation

Read results in emission units (X). Subtract blank emission from all samples (K blank should read 0.00 to 0.02; Na blank should read 0.00 to 0.025). Calculate the Na/K concentration (Y) by using the power curve fit formula, $Y = ax^b$, as below. For power curve fits, both X_i and Y_i must be positive. Convert Na/K concentration to desired units, i.e., percent, ppm, mg/100, etc., using the appropriate conversion factors.

Power curve fit:

(Concentration) Y

$$Y = ax^b$$

x(emission)

$$b = \frac{\sum (\ln X_i)(\ln Y_i) - \frac{(\sum \ln X_i)(\sum \ln Y_i)}{n}}{\sum (\ln X_i)^2 - \frac{(\sum \ln X_i)^2}{n}}$$

$$a = \exp [\sum \ln Y_i - (b) (\sum \ln X_i)]$$

Where:

X_i = emission of each standard

Y_i = concentration of each standard

$$\sum = \sum_{X_i, Y_i}^{X_n, Y_n}$$

n = number of standards used

\ln = log normal

Y = sample concentration

X = sample emission

Ref: Official methods of Analysis of the Association of Official Analytical Chemist, 14th Edition.

PART 5—CHEMICAL RESIDUE PROCEDURES

5.001 Chlorinated Hydrocarbon Analysis

Because chlorinated hydrocarbon pesticides are stored in fat, or fatty tissue and tolerances are set for these residues in fat, all such analyses are reported on the basis of the extracted or rendered fat.

5.001A. Theory

The sample is dissolved in petroleum ether and extracted with acetonitrile. The acetonitrile extracts are diluted with water to partition the pesticide residues into petroleum ether. The residues are purified by chromatography on a Florisil column, eluting with a mixture of petroleum and ethyl ethers. Residues in the concentrated eluates are measured quantitatively by gas-liquid chromatography or semi-quantitatively by thin layer chromatography. Identification is by a combination of gas-liquid and thin-layer chromatography.

5.001B. Apparatus

- (a) Chromatographic columns: 22mm id \times 300 mm with teflon stopcocks and coarse fritted plate.
- (b) Alternate Column: Chromatographic columns, 22mm id \times 300 or 400 mm without stopcocks. With coarse fritted plate. - (when this column is used, it is topped with anhydrous sodium sulfate powder for flow control).
- (c) Filter tubes: Approximately 22mm \times 200mm with a short delivery tube and a coarse fitted plate (a glass wool plug can also be used).
- (d) Kuderna-Danish Concentrators: Five hundred ml with 5 or 10 ml volumetric receiving flasks or graduated tubes (Kolmer or Mills types; Kontes Glass Company, Vineland, New Jersey 08360, Catalog No. K-570000, K-621400, or equivalent.)
- (e) Alternate concentrator: If the solutions can be made to a volume of 25 ml, an Erlenmeyer flask with a 24/40 standard taper joint with a three ball Snyder column may be used.
- (f) Separatory funnels: Teflon stopcocks - 500 ml and 125 ml.
- (g) Thin layer apparatus and accessories: Desaga/Brinkman apparatus for thin layer, or equivalent.
- (h) Chromatographic tank and accessories: Arthur H. Thomas Co., Vine Street at 3rd, Philadelphia, PA. 19105, No. 3106-FO5, or equivalent, with metal or glass troughs.
- (i) Ultra-violet light source: Four germicidal lamps, 15 watt, 18" long mounted in a metal cabinet 20" \times 10" \times 10" to accommodate two glass plates.
- (j) Gas liquid chromatograph: Consisting of an electron capture detector, power supply, electrometer and 1 mv recorder, on-column injection system, all-glass column in oven controlled to $\pm 0.1^\circ$ C.
- (k) Column: Six feet \times four mm id (approximately) packed with 10 percent OV-101 on 100-120 mesh Anakrom ABS. (Analabs Inc., Post Office Box 501, North Haven, Connecticut 06473). The column should be conditioned at 250° C with a nitrogen flow of 100 to 120 ml/min until endrin emerges as a single peak.

(1) Alternate columns 6-ft \times 4 mm (approximately) packed with 1.5 percent OV-17 and 1.95 percent OV-210 on 100-120 mesh Chromsorb VHP or equivalent, or 1.0 percent OV-17 and 4.0 percent OV-210 on 100-120 mesh Gaschrom Q. The column should be conditioned at 250° C. Other equivalent liquid phases and support may be used. (If this column is used, the 6 percent and 15 percent elutions may be combined).

5.001C. Solvents

Some solvents, sold as suitable for use as received, are available from many manufacturers, and are usually identified as Nanograde or chromatographic grade. All solvents, however, must be tested prior to use to determine whether purification is required. This test is to be performed as outlined on the following page. Those solvents requiring purification must be distilled in an all-glass apparatus. However, if Nanograde or chromatographic grade solvents are found to require purification, they should be returned to the supplier with a request for replacement since their high cost is predicated upon purity.

(1) CAUTION

Ethers (excluding petroleum ether) containing unsafe concentrations of peroxides can detonate when they are distilled or concentrated. No quantity of ether, therefore should be distilled or concentrated before the following test is performed:

"Shake 1 ml of a freshly prepared saturated solution of KI with 9 ml of ether in a 25 ml glass stoppered cylinder. Any yellow color indicates a concentration of peroxide greater than 0.005 percent."

Such a concentration is dangerous, and ether containing this level of peroxide should be discarded, or returned to the supplier with a request for an exchange. Prior to discarding or returning the ether should be deactivated in the following manner:

"Add 30 ml of a 30% (w/v) aqueous ferrous sulfate solution per liter of ether. Use caution, because this reaction may be vigorous if ether contains high concentration of peroxides."

(2) PURITY TEST

Electron capture gas chromatography requires the absence of substances causing any detector response from the following test: Place 300 ml of solvent in the Kuderna-Danish Concentrator fitted with a three-ball Snyder column and a calibrated collection tube and evaporate to 5 ml. Inject 5 μ l of concentrate into the gas chromatograph using operating conditions described in the paragraph, "Residue Detection Method - Gas Chromatograph." Concentration must not cause recorder deflection greater than 1.0% from base line for 2 to 60 minutes after injection. If thin layer is used for semi-quantitative analysis, 50 μ l of the concentrate is spotted on the plate, developed, sprayed and exposed to UV light as described in the paragraph (5.001H). "Thin-layer chromatography." There should be no spots visible.

5.001D. Reagents

(a) Acetonitrile: Practical Eastman Organic Chemicals (our experience has been that it seldom requires purification). If purification is necessary, purify as follows:

To four liters of acetonitrile add 1 ml of H_3PO_4 , 30 grams P_2O_5 , a few boiling chips and distill in all glass apparatus at 81-82° C. DO NOT EXCEED 82° C.

(b) Acetonitrile saturated with petroleum ether: Saturate CH_3CN with petroleum ether (c). A small layer of petroleum ether should just be visible on the CH_3CN .

(c) Eluting solvent, 15 percent: Dilute 150 ml ethyl ether (d) to one liter with petroleum ether (c).

(d) Ethyl ether: Reagent or ACS grade. (See Caution under Solvents).

(e) Petroleum ether: 30° - 60°C. boiling range.

(f) Sodium sulfate: Anhydrous granular and anhydrous powder.

(g) Aluminum oxide: Al_2O_3 -G available from Warner-Chilcott Laboratories, Instruments Division, 200 South Garrad Blvd., Richmond, California 94801. Al_2O_3 -G neutral, available from Merck & Co., Rahway, New Jersey 07065.

(h) Developing Solvents

(1) n-Heptane, commercial or technical grade (no purification required)

(2) n-Heptane, commercial or technical grade, containing 2 percent acetone, reagent grade.

(i) Chromogenic agent: Dissolve 0.100 grams AgNO_3 in 1 ml H_2O , add 20 ml 2-phenoxyethanol (Practical Eastman Organic Chemicals), dilute to 200 ml with reagent grade acetone, add one small drop of 30 percent H_2O_2 and mix. Store in dark over night and decant into spray bottle. Discard after four days.

(1) Standard solutions for thin-layer chromatography.

a. Stock solutions A_1 and A_2 (mixture of aldrin, lindane heptachlor epoxide and methoxychlor): Weigh 0.1 g of each pesticide into same 10 ml glass stoppered volumetric flask (A_1). Repeat with 0.05 g of each into a second volumetric flask (A_2). Dissolve in ethyl acetate, dilute to 10 ml and mix. One μl of each solution contains 10 μg and 5 μg , respectively, of each pesticide.

b. Stock solutions B_1 and B_2 (mixture of BHC, DDE, DDD, DDT, dieldrin, and endrin): Prepare solutions of 10 and 5 $\mu\text{g}/\mu\text{l}$ as in (a).

c. Dilutions of stock solutions: From the stock solutions prepare dilutions containing 0.2, 0.1, 0.05, 0.02, 0.01, 0.005 and 0.002 $\mu\text{g}/\mu\text{l}$.

(j) Individual Standards: Separate standards of each insecticide should be prepared at 0.1 $\mu\text{g}/\mu\text{l}$ concentration so that the migration of each insecticide can be determined and the insecticide can be identified in the mixtures.

(k) Gas-liquid chromatography: The individual standards from "(j)" are diluted so that a μl injection will contain approximately 0.05 nanogram of heptachlor epoxide and will give approximately 50 percent of full scale deflection on the recorder. The concentrations of all other insecticides should be adjusted accordingly: Lindane and BHC should be approximately 0.5 of the heptachlor epoxide; dieldrin, endrin and DDE should be 1.5 times; DDD should be about 2 times, DDT about 4 times and methoxychlor about 8 times. This is approximate, and must be determined for each gas-liquid chromatograph used.

(l) Gas liquid chromatography mixture standards: After retention time and response have been determined for the separate insecticides, prepare mixtures so that the peaks will not interfere with each other, i.e., BHC, DDE, DDD and methoxychlor in one solution; lindane, aldrin, heptachlor epoxide, dieldrin and endrin in another. The concentrations should be such that a 5 μl injection produces a 1/2 scale recorder deflection.

(m) Florisil: 60/100 Pesticide Residue (PR) grade, activated at 1250°F (675°C) available from the Floridin Co. When 1250° F activated, Florisil is obtained in bulk, transfer immediately after opening to ca 1 pt (500 ml glass jars, or bottles, with glass-stoppered or foil-lined, screw-top lids, and store in dark). Heat ≥ 5 hours at 130° C before use. Store at 130° C in glass-stoppered bottles or in a desiccator at room temperature and reheat at 130° C after two days.

5.001E. Standardization of Florisil

The columns are prepared as in the clean up technique, and prewashed with 100 ml petroleum ether. A 1 ml aliquot of mixed pesticides containing 0.2 μg lindane, 0.4 μg heptachlor epoxide, 0.6 μg dieldrin and 0.6 μg endrin is placed on the column. The columns are eluted as in the clean up technique. The eluate is concentrated, transferred to 25 ml

volumetric flasks and made to volume with petroleum ether. One ml of the mixed pesticides is also diluted to 25 ml with petroleum ether in a volumetric flask. Inject 5 μ l of the standard and of the eluate into the gas chromatograph. The recovery of all the pesticides when compared to the standard must be at least 95 percent when the 15 percent eluate is used.

5.001F. Determination

5.001F.1. Sample Preparation

(a) *Fatty Tissue*

Pass chilled sample through food chopper twice. If dry ice is available, add small quantities to fat to maintain a low temperature. Plug a powder funnel moderately tight with glass wool; place approximately 100 g of the comminuted fat in the funnel; position the funnel in a beaker and render at 100-110°C until the fat ceases to drop.

(b) *Muscle Tissue*

Pass sample through food chopper three times. Grind suitable quantity with anhydrous Na_2SO_4 to combine with moisture in sample. Transfer granular mixture to centrifuge bottle, add 100 ml petroleum ether, shake vigorously and centrifuge about 5 minutes at about 1500 rpm. Pour off solvent layer into beaker and re-extract residue twice with 50 ml portions of petroleum ether. Evaporate combined extracts to obtain fat.

All chlorinated hydrocarbon residues are reported on the basis of the rendered or extracted fat.

5.001F2. Acetonitrile Partitioning

(a) Weigh 3 g of melted fat into a 50 ml beaker, add 10 ml of petroleum ether and transfer to a 125 ml separatory funnel. Fortify each sample with an amount of Aldrin as an internal standard which results in an approximate 50 percent scale deflection. Note: An enhanced peak for Aldrin may indicate the presence of Ronnel.

(b) Use an additional 15 ml in small portions to rinse the beaker for a quantitative transfer of the fat.

(c) Add 25 ml of acetonitrile saturated with petroleum ether and shake vigorously for 1 minute.

(d) Let layers separate (adequate time must be allowed to obtain a good separation).

(e) Drain the bottom (acetonitrile) layer into a 500 ml separatory funnel containing 300 ml of 4 percent Na_2SO_4 and 100 ml of petroleum ether.

(f) Extract the petroleum ether solution in the 125 ml separatory funnel with 3 additional portions of acetonitrile saturated with petroleum ether, shaking vigorously 1 minute each time. Combine all extracts in the 500 ml separatory funnel.

(g) Stopper, invert the 500 ml separatory funnel, vent pressure, and shake gently 1 minute to transfer the pesticides to petroleum ether.

(h) Let layers separate cleanly and discard lower aqueous layer.

(i) Caution: occasionally excessive pressure will build up. Wash the petroleum ether in 500 ml separatory funnel with two 50 ml portions of 4 percent Na_2SO_4 , stopper invert and after each addition vent.

(j) Discard washings and draw off petroleum ether layer through a 2 inch column of anhydrous granular Na_2SO_4 in the filter tube into a 250 ml erlenmeyer flask.

- (k) Rinse the separatory funnel and the column with two 10 ml portions of petroleum ether.
- (l) Evaporate combined extract and rinses to ca 10 ml in a Kuderna-Danish or alternate concentrator.

5.001F3. Florisil Column Chromatography

- (a) Prepare 22 mm id Florisil column, containing 100 mm after packing of activated Florisil topped with about 1/2 inch of anhydrous sodium sulfate powder (110 mm should pack to 100 mm).
- (b) Prewet column with 40-50 ml petroleum ether.
- (c) Transfer petroleum ether extract to column, letting it pass through the column at a rate not to exceed 3 ml/min (optimum rate is approximately 2.5 ml/min).
- (d) When the last of the petroleum ether has sunk into the column, change receivers and elute with 200 ml 15 percent ethyl in petroleum ether and proceed to step (e).
- (e) Concentrate each eluate to 5 ml using the Kuderna-Danish or alternate concentrator. Transfer each eluate quantitatively to a 25 ml volumetric flask and make to volume with petroleum ether. When the elution from the florisil column is held to the low rate specified, the insecticides are eluted quantitatively from the column and both eluates are suitable for thin-layer or gas-liquid chromatography.
- (f) The 15 percent eluate contains aldrin, BHC, DDE, DDD(TDE), o,p-DDT and p,p'-DDT, heptachlor, heptachlor epoxide, lindane, methoxychlor, HCB, Mirex, oxychlordane, dieldrin, and endrin.

5.001G. Residue Detection Method Gas Chromatography

Operating conditions for 10 percent OV-101 or the mixed phase column. Injection temperature 225° C, column temperature 185° C to 200° C, detector temperature 210° C (maximum if H³ detector is used), carrier gas flow 120 ml nitrogen/min. Detector voltage, instrument settings must be determined for each chromatograph to obtain the maximum sensitivity.

Check the linearity of the system. Determine the individual retention times of the insecticides. *Make calculations only from peaks that are in the linear range.*

Inject a suitable aliquot (3-8 µl) of the eluates from the 25 ml volumetric flask into the chromatograph using a 10 µl syringe. If peaks are out of the linear range, re-inject using a smaller aliquot or dilute to bring the response into the linear range. Tentatively identify residue peaks on the basis of retention time. Measure areas under the peak or peaks from the unknown and compare to the areas obtained from known quantity of the standard insecticide. For most accurate measurement, unknown and standards should be of similar size (i.e. ±15%) and in the linear range of the detector used.

Chromatograph the standard pesticide immediately after the sample. Alternatively, it is possible to use only peak heights with a slight loss in accuracy, but good judgement is required, and it is *very* important that all measurements be in the linear range of detector.

5.001H. Thin-Layer Chromatography

- (a) *Preparation of adsorbent layer* (TLC plates are commercially available)

Select five 8 × 8 inch double strength window glass plates of uniform width and thickness and smooth off the corners and edges with a file or other suitable instrument. Before coating, wash the plates in hot soapy water and thoroughly rinse with distilled water. Press the plates snugly into position on the mounting board which has a retaining ledge on one side and one end. The plastic board is mounted so that its long side with raised ledge faces the operator while the short side with ledge is to the right of the operator. Before coating, the surface of the plates are wiped with a few ml of 95 percent ethanol on a tissue to remove fingerprints or other adhering material.

Position the applicator (set at 250 microns). trough open, with the left edge 1/4 inch in from the edge of the first plate to be coated.

To coat 5 plates, weigh 30 g $\text{Al}_2\text{O}_3\text{-G}$ into a 250 ml standard taper Erlenmeyer flask. Add 50 ml distilled water, stopper flask and shake moderately for 45 seconds. Violent shaking produces bubbles resulting in a "pockmarked" layer.

NOTE: Suspensions which contain adsorbent with binders set rapidly and the entire procedure from the preparation of the slurry to the final coating must be completed within 2 minutes.

After shaking, immediately pour the slurry into the applicator chamber. Rotate the chamber by turning the handle through 180° . After a few seconds, the slurry begins to flow out of the exit slit. Grasp the applicator with both hands and pull it manually with a steady motion across the series of plates. Approximately 5 seconds are required for the actual coating procedure. Immediately after application, tap the edge of the mounting board or shake the entire board gently to smooth out slight ripples or imperfections in the wet coating.

Let coated plates dry in position on mounting board for 15 minutes. Then dry plates in a forced draft oven at 80°C for 30 minutes. Remove plates and cool.

Examine the plates carefully in transmitted and reflected light for imperfections or irregularities in the coating. Plates should be discarded if extensive rippling or mottling of the layer is observed.

Five more $8'' \times 8''$ plates may be prepared while the first set is drying. Be sure the applicator is thoroughly cleaned and dried before reusing. The 10 coated and dried plates may be prewashed immediately.

(b) *Prewashing or Adsorbent Layer* (Not necessary for commercial plates)

Scrape 1/2 inch of adsorbent off the one edge of the plate at 90° to direction of application with a razor blade. Then pour 15 ml 50 percent aqueous acetone into a metal trough inside the chromatographic tank. Cut out a $3/4$ inch \times 8 inch strip of Whatman No. 1 filter paper, wet with solvent and place over the scraped off portion with 1/4 inch overlapping the adsorbent layer. Place the plate in the chromatographic tank, seal tank with masking tape if necessary and develop with 50 percent aqueous acetone to within 1-1/2 inches from the top of the plate (75-90 minutes). Remove the plate from the tank, remove filter paper wick, invert the plate, and dry in the hood for 5 minutes. Dry the plate at 80°C for 45 minutes. Remove plate from oven, cool and store in desiccator until needed. The prepared plates should be used within one week after preparation.

(c) *Sample Spotting*

A pencil mark is made 1-1/2 inches from the bottom of the plate (edge with adsorbent removed) at both sides. An imaginary line between the two points indicates the sample spotting or origin "line." Draw a line (which removes the coating) completely across the plate 5-1/2 inches from the bottom edge. This line represents the solvent front after development. On the lower edge of the adsorbent starting $3/4$ inch in from the left edge of the plate make 18 marks with a pencil at $3/8$ inch intervals. (Fewer marks with greater intervals may be used if desired). The marks serve as horizontal guides to sample application. The identity of samples and standards may be etched directly into the adsorbent layer above the solvent front line and in alignment with these marks.

The imaginary spotting "line" is actually a shadow line cast by a strong light source from a wooden ruler supported 1 inch above the plate. The ruler shadow is aligned on the two 1-1/2-inch marks on either edge of the plate. The shadow line and 18 marks respectively serve as vertical and horizontal guides for sample application.

For optimum semi-quantitative estimation, adjust aliquot of sample spotted to give residue spot within the range $0.005\text{ }\mu\text{g}$ to $0.1\text{ }\mu\text{g}$. Concentrations of standards and mixtures spotted should be 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, and $0.2\text{ }\mu\text{g}$. Sample spots greater than $0.2\text{ }\mu\text{g}$ are difficult to estimate quantitatively and less than $0.005\text{ }\mu\text{g}$ may be difficult to distinguish. The volume of sample extract spotted should not exceed $10\text{ }\mu\text{l}$ if possible and spotting should be done repeatedly with a $1\text{ }\mu\text{l}$ Kontes spotting pipet, or equivalent. Care should be taken not to disrupt the surface of the adsorbent. Standard and sample solutions should be spotted with the same pipet, but the pipet must be cleaned between spottings. For the best results keep the size of spotted sample as small as possible.

Spot standard solutions of pesticides (mixtures A&B or single compounds) in different positions on the same plate. The choice of pesticides to be used for identification may be governed by the pesticide residues to be expected in the sample examined. For semi-quantitative estimation, varying amounts of knowns may be chromatographed from spots adjacent to samples.

(d) *Development, Spraying and Exposure of Plates*

1. *Development*

Place liners and trough in tank. Presaturate liner by pouring 75 ml of the appropriate developing solvent into bottom of tank prior (30 minutes or longer) to developing plate. Presaturation decreases development time and improves uniformity of F_r values.

For plates spotted with 6 percent extracts, pour 50 ml n-heptane into the trough. Place the lower edge of the plate in the trough (a wick may be necessary) with the top of the plate leaning against the side of the tank. Place the glass cover plate on the tank and seal with masking tape.

For plates spotted with 15 percent extracts use heptane-acetone (98 + 2) as developing solvent.

When the solvent front just reaches the pencil line 10 cm above the spotting "line," remove the plate and dry in hood for 5 minutes.

2. *Spraying*

Support plate on one side and spray fairly heavily with the chromogenic reagent, using lateral motions of the spray bottle perpendicular to the direction of solvent flow.

Spray until plate appears translucent or soaked with reagent. Underspraying will result in poor sensitivity. After spraying, dry the plate in the hood for 15 minutes, then place immediately under ultraviolet light source.

3. *Exposure* (Warning! Protect eyes from ultraviolet light)

Expose the plate to ultraviolet light until the spot for the standard of lowest concentration appears. Five nanogram quantities of most of the chlorinated organic pesticides should be visible after 15-20 minutes exposure. Exposure times in excess of 30 minutes will not harm the plates. For best results place plates 3" from the bottom edge of the germicide tubes.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

5.002 MICRO ALUMINA COLUMN SCREENING ANALYSIS FOR CHLORINATED HYDROCARBONS AND PCB RESIDUES IN FATTY FOODS

5.002A. Theory

Chlorinated Hydrocarbons are extracted and separated by elution from fat in small glass columns filled with partially deactivated alumina. The eluate is carefully evaporated to a workable volume and an aliquot injected into a gas chromatograph for detection and quantitation.

5.002B. Apparatus

- (a) 50 ml (or other suitable size) ground glass (19/22) Erlenmeyer flask - Kontes #K-296510, or equivalent.
- (b) 19/22 ground glass toppers - Kontes #K-850500, or equivalent
- (c) Micro column — 200 ml plain chromaflex columns with Teflon stopcock (Kontes #K-420280-213) or 12 × 300 mm fritted glass columns with Teflon stopcock, or equivalent
- (d) Glasswool (borosilicate or silanized)
- (e) 10 ml, leur-lok syringe with 2 or 4 inch 17 gauge needle
- (f) Gas chromatograph - equipped with electron capture detector and a column of choice having at least 1,500 theoretical plates for aldrin and the ability to separate all compounds of interest.
- (g) Explosion proof blender (Warning Model E-1), or equivalent
- (h) Concentrator tubes (Kdurna Danish, or equivalent)
- (i) 25 ml volumetric flask

5.002C. Reagents

(a) Alumina-Fisher Alumina (catalog #A950) Wolm Alumina, or ICN Alumina (catalog #408420) neutral, Brockman Activity I, 80/200 mesh, or equivalent. Treat alumina in an 800°C furnace for a minimum of 4 hours in an open container. Store in an 130°C oven in an open container. Deactivate by cooling to room temperature in capped container, adding a percentage of distilled water, the percentage of which has been predetermined to give an 80-110 percent recovery through the column of the chlorinated hydrocarbons of interest, by weight; recap and shake vigorously, allow to equilibrate overnight or at least for 4 hours before use. Deactivate only the amount to be used each day.

(b) p—methoxyazobenzene, (4 phenylazo-anisole); Columbia Organic Chemicals Co., Inc., Columbia, SC 29220, or equivalent - Dissolve 0.3 g in 100 ml hexane.

- (c) Hexane - distilled in glass, Burdick and Jackson, or equivalent
- (d) Acetone - distilled in glass, Burdick and Johnson, or equivalent
- (e) Petroleum Ether - distilled in glass, Burdick and Jackson, or equivalent
- (f) "Blank" fat
- (g) Standard chlorinated hydrocarbon solutions, (concentration as appropriate for instrumentation and samples being tested)

5.002D. Sample Preparation

(a) For high fat content samples - grind or chop and heat render at 100°-110°C.

(b) For low fat content samples - grind and solvent extract by blending approximately 200g sample with 1 + 1 acetone/petroleum ether in an explosion-proof blender. Filter tissue and solvent through glass wool plug in a funnel into a 400 ml beaker and allow layers to separate. Decant petroleum ether layer containing the fat into 100 ml beaker. Add a few glass beads and evaporate solvent on a steambath.

5.002E. Column Preparation

Fill the glass columns, 5.002B. (c), fitted with small glass wool plug, depending on column type, 3/4 full with petroleum ether or hexane. Add 10 g of prepared alumina and allow to settle. Then drain just to top of alumina in column. Collect petroleum ether or hexane in a suitable size Erlenmeyer flask. Rinse flask with this solvent and discard. Replace flask under column.

5.002F. Determination

NOTE: Steps (a)–(e) may be eliminated, but there is a danger of ruining the column in the gas chromatograph if this is done. It is preferable to conduct steps (a)–(e) one time with each set of samples.

(a) Determine amount of elution solvent necessary to separate chlorinated hydrocarbons from the lipids in the sample by dissolving 1 ml of dye solution 5.002C.(b) in 0.1 -0.2g “blank” fat.

(b) Transfer this dyed “sample” to a prepared column with small portions, totaling 5 ml, of petroleum ether or hexane with a syringe.

(c) Allow to drain into column with a flow rate of one drop per second, collecting eluate in receiving flask.

(d) Fill the column with more petroleum ether or hexane as initial 5 ml penetrates into alumina and adjust flow rate to 1 drop per second.

(e) The visible dye will separate from the fat as it flows down the column in a bright orange band, which may disperse slightly near the bottom. When this band has eluted, measure the volume required to elute the dye band. This is the amount usually sufficient to elute the chlorinated hydrocarbons from the column separated from the fat. (Unusual amount is approximately 35 ml.) Slightly more elution solvent may be required for some chlorinated hydrocarbons.

(f) Quantitatively weight 0.1-0.2 g fat into aluminum dish or small beaker, or weigh 2.5 g rendered fat into 25 ml vol flask and dilute to volume with hexane or petroleum ether. For quality control purposes add a known amount of aldrin to each sample as an internal standard.

(g) Rinse fat sample (or 1 ml of fat solution prepared in step (f)) into a prepared alumina column with syringe using about 5 ml petroleum ether or hexane and allow to drain to near the alumina surface with care not to allow column to go dry.

(h) Rinse sample beaker or dish with additional petroleum ether or hexane and add rinses to column. Collect eluate in a suitable size flask for volume determined in step (e). The rinses comprise part of the predetermined solvent volume. If a concentrator tube with an attached reservoir (Kduma Danish) is used to collect the eluate, the transfer in step (j) may be avoided.

(i) After elution of column rinse tip of column and neck of flask with a small amount of petroleum ether or hexane using syringe.

(j) Evaporate solvent to 2-3 ml using steam bath. Transfer to a concentrator tube and concentrate nearly to dryness using a stream of filtered nitrogen or air. Do not use heat, and do not allow sample to go to dryness. If residue shows enough lipid material to cause chromatographic problems, start over do not go to step (k).

(k) Dilute residue with 1-4 ml hexane or petroleum ether and inject into appropriate gas chromatograph for detection and quantitation.

Reference

Claeys, Robert R. and Inman, Roderick, D.; JAOAC, 57 (1974)

5.003 Gel Permeation Chromatography Method for Determining Organochlorine Pesticides in Animal Fats

Liquified animal fat is dissolved in methylene chloride:cyclohexane (1:1) solvent. Residues are purified by gel permeation chromatography (GPC). Residues in the concentrated eluates are identified and measured by GLC-EC detection. This method has been collaboratively studied with acceptance by both AOAC and FSIS.

Compounds to Which Method has been collaborated

Aldrin	Dieldrin	Heptachlor
BHC	o,p'-DDT	Heptachlor Epoxide
Lindane	p,p'-DDT	Hexachlorobenzene
cis-chlordane	p,p'-DDE	Methoxychlor
trans-chlordane	p,p'-TDE	Mirex
	Endrin	
	Oxychlordane	

Compounds to Which Method Applies

Polychlorinated Biphenyls
Polybrominated Biphenyls
Toxaphene
Hallowaxes

5.003A. Apparatus and Supplies

(a) Gel Permeation Chromatographic System (GPC). AutoPrep 1002A Gel Permeation Chromatograph, Analytical Bio-Chemistry Laboratories, Inc., P.O. Box 1097, Columbia, Missouri 65205 or equivalent LC system.

(b) Solvent concentration apparatus. Rotary evaporation system, such as by Calab, Emerville, CA or equivalent with 30°C water bath.

(c) Gas Chromatograph - EC Detector (⁶³Ni) See AOAC *Official Methods* Sec. 29.008.

(d) Convection Oven: Able to maintain a temperature of 100°C ± 1°C.

(e) Solvents: Glass distilled of residue analysis quality.

(f) Bio Beads SX-3 resin, 200-400 mesh, Analytical Biochemistry Laboratory, Inc., Box 1097, Columbia, MO 65205.

(g) Pear shaped flask, 100 ml. Kontes.

(h) Analytical Standards, EPA.

(i) Volumetric class 'A' flask 10 ml.

(j) Reverse flow chromatography column 2.5 × 60 cm Kontes.

5.003B. Preparation of Sample

(a) Place approximately 40 g representative animal fat sample in glass funnel (8.0 cm) with glass wool plug. Place funnel in flint glass bottle or 250 ml beaker in oven ≤ 100°C until 5-10 g fat has been collected. Mix thoroughly.

5.003C. Column Preparation

- (a) Place 60 grams of BioBeads SX-3 into a 500 ml beaker.
- (b) Cover the resin with at least 2 inches of methylene chloride:cyclohexane (50:50) mixture and allow it to stand overnight to swell the gel.
- (c) Decant off fines, add fresh solvent allow to stand 30 minutes and again decant off fines. Repeat two more times.
- (d) Secure the upper plunger assembly (short plunger) in the tube by inserting the plunger and turning it clockwise until snug.

CAUTION: This end of the column is the exit and the plunger should not be loosened again. Adjustment after the resin is packed may result in wrinkling the Teflon cloth seal and allow BioBeads to escape into the instrument.

- (e) Clamp the chromatography tube upside-down on a ring stand and place the line from the upper plunger assembly into a waste beaker *below* the column.
- (f) Transfer the swelled resin to the column as a slurry with the solvent mixture.
- (g) Allow the resin to settle and the excess solvent to drain from the column until the level of solvent and BioBeads is equal.
- (h) Insert the other plunger assembly (the long plunger) into the tube until it touches the gel and tighten the plunger by turning it clockwise until snug.
- (i) Connect the column input and output lines together and install the column on to pumping system.
- (j) Connect the column input line to the correct line from the sample introduction valve.
- (k) Place a glass beaker under the column output line.
- (l) Turn the pump on and allow it to run until solvent runs out the column output line.
- (m) Hold the glass beaker with the column output line in it up to a light and check to insure that no BioBeads are escaping from the column.
- (n) If the column has voids or air pockets after pumping for 230 minutes, loosen the inlet plunger (lower long plunger) and compress the column.
- (o) Retighten the inlet plunger and continue pumping. Normal pressure is about 5-15 psi at a flow of 5 ml/min.

5.003D. Column Calibration

The elution volumes in Appendix I are used as a guide to determine approximately what volumes to collect during fractionation. It is recommended that the volume of eluant from 150 ml to 320 ml be fractionated to insure residue collection. An initial dump time of 30 min followed by seventeen 2 minute collect times will provide a margin before and after the usual elution volume. To autofractionate, proceed as follows:

- (a) Turn the "power on/off" switch on the control module to the "on" position.
- (b) Press the "pump enable" switch to start the pump set at a flow of 5 ml/min.

(c) Set the "terminal loop" thumbwheel switch to "17" and place collection vessels under the appropriate numbered sample collection tubules.

(d) Set the "wash" time switch to "00".

(e) Set the "collect" time switch to "02" with the "time base" switch set at 1 minute.

(f) Set the "dump" time switch to "30".

(g) Turn the "pump on/off" switch to "on".

(h) Depress the "reset" switch until the "current loop" display reads "00".

(i) Press the "index" switch to "01" on the "current loop" display.

(j) Rotate the "load/run" valve on the cabinet counter clockwise to the "load" position.

(k) Inject a minimum of 8 ml of the sample (suspended in GPC solvent) into loop #1 through the sample introduction port with a 10 cc syringe.

(l) "Index" to loop "02" with the syringe still in position.

(m) Remove the syringe and rinse with clean solvent.

(n) Rinse loop "02" and connecting lines by injecting 8 ml of clean solvent.

(o) Push the "reset" switch until "00" is displayed on the "current loop" display. Be sure all loops are full of solvent.

(p) Rinse loop "00" with 8 ml of clean solvent, rotate the "load/run" valve to "run", remove the syringe, and depress the "auto start switch". The "dump" time of 30 min is loaded into memory.

(q) Now set the "dump" time switch to "00". The AutoPrep is now programmed to dump 150 ml and collect 170 ml in 10 ml fractions.

(r) The solvent in each 10 ml fraction can then be evaporated and brought up to 1 ml volume in iso-octane for a qualitative GLC-EC determination. After determining which fractions contain organochlorine pesticides, the collection volume must be decided. Only a small percentage of BHC (lindane) should elute from the column in the 160 ml to 170 ml fraction (shown in Appendix I). In an attempt to limit the amount of lipid in the collected fraction, collection should begin as late as possible. This will allow more of the "late" eluting lipid to be dumped before collection begins. Finally, a 5 ml margin is added after the last residue containing fraction to further ensure quantitative recovery.

5.003E. Sample Chromatography

(a) Weigh 2.0 g fat into a 10 ml volumetric flask. Fortifications are made at this point in the procedure (standards in $\text{CH}_2\text{Cl}_2:\text{C}_6\text{H}_{12}$). Dilute to 10 ml with $\text{CH}_2\text{Cl}_2:\text{C}_6\text{H}_{12}$ (1 + 1) and mix thoroughly. For screening purposes the fat sample is weighed directly into 16 × 125 mm disposable glass culture tubes. Solvent is added to a pre-determined 10 ml mark, tube is capped with a teflon lined cap and mixed by shaking.

(b) Centrifuge or filter the sample if particulate matter is visible.

(c) Fill the 5 ml GPC sample loop with 7 ml of sample.

(d) Process samples using dump/collect times determined in the calibration section.

(e) Collect the "collect time" eluent in a 100 ml pear shaped flask and rotary evaporate to near dryness at $\leq 30^{\circ}\text{C}$. For screening purposes samples may be concentrated on a steambath with 2" diameter opening and teflon chips as boiling stones. Flasks are heated by raw steam and solvent concentrated to 2-5 ml.

(f) Transfer residue quantitatively to a final volume of 5 ml.

5.003F. Residue Quantitation by Gas Chromatography

(a) Residues are chromatographed on OV-101 or OV-17, OV-210 columns. All residues of interest must be 85 percent resolved for quantitation. Chromatographic parameters should be set such that a 0.10 ng injection of Aldrin yields at least a 90 percent full scale deflection with a retention time of 2.3 ($\pm .07$) minutes. Compounds of interest must not be degraded by any part of the GC system. For screening purposes aldrin may be added after solvent evaporation and used as an internal standard for GC quantitation purposes.

(b) Centrifuge or filter with Millipore LC 10 filters if particulate matter is visible. Use 7 ml sample to load sample loops on precalibrated GPC. A 5 ml aliquot is accepted into the sample loop. Process through GPC using dump/collect times from fractionation procedure and collect eluant in 100 ml pear shaped flask. Rotary evaporate to just dry at $\leq 30^{\circ}\text{C}$. Transfer residue quantitatively with 10 ml iso-octane or equivalent GLC-EC compatible solvent. Adjust volume under gentle, dry N_2 stream to 5.0 ml.

Gas Chromatography

Any GLC and column which separates for quantitation all compounds of interest (see AQ critical points) or equivalent is recommended. The instrument consists of on-column injection system, all-glass column in oven controlled to $\pm 0.1^{\circ}\text{C}$, electron capture detector with independent power supply, electrometer, and appropriate mV recorder.

The injection time should be greater than the retention time for methoxychlor. Compounds of interest must be degraded by any part of the GLC system. A 3-6 μl injection containing 0.120 nanograms of heptachlor epoxide in iso-octane should give approximately 90 percent full scale deflection. The concentrations of the other organochlorine pesticides in the standard solution should be adjusted to produce recorder deflections similar to heptachlor epoxide. The retention time for aldrin should be 2.3 min ($\pm .07$). If a particular compound is known to be a problem, the instrument may be sensitized for optimal response to that specific compound. The peak recognition window should be $\pm 3\%$ of the compounds retention time.

Electron Capture Detector

Operate ^{63}Ni electron capture detector to produce stable, reproducible, linear response, and adjust amount of injected sample to accommodate difference in instrument sensitivity. Desirable peak size for quantitation should exceed 100 mm if peak height is used and 10,000 to 100,000 counts if peak area is used, integrated at 1 count/v-sec. The linear response of the detector should be checked frequently. The injector temperature should be at 250°C while the detector temperature is 350°C .

APPENDIX I

Organochlorine Pesticide's Elution Profiles Through 60 g SX-3 Styrene Divinylbenzene Gel, ca 48 cm Bed Length × 2.5 cm column diameter.

Organochlorine Pesticide	Elution Volume (ml) with $\text{CH}_2\text{Cl}_2:\text{C}_6\text{H}_{12}$ (1:1, v:v)
Aldrin	160-240
alpha BHC	180-240
gamma BHC (Lindane)	170-240
cis Chlordane	160-250
trans Chlordane	160-230
Dieldrin	160-230
p,p'-DDE	160-220
α,p'-DDT	160-220
p,p'-DDT	160-230
Endrin	160-220
Heptachlor Epoxide	160-230
HCB	190-240
Methoxychlor	160-230
Mirex	160-230
Oxychlordane	150-200
Toxaphene	160-230

5.004 Confirmatory Method for Chlorinated Hydrocarbons

This is a confirmatory method using a capillary column for separation and electron impact ionization for detection. The method is capable of confirming twelve chlorinated hydrocarbons at concentrations as low as 10-80 percent of the action level using the extract from the GPC, Micro Alumina Column, or Florisil Column method. The absolute sensitivity of the confirmatory method varies from compound to compound. The method has not been validated. Confirmation generally requires injection of approximately 5 percent of the sample extract.

5.004A. Theory

Selected ion monitoring is used to confirm the presence of the twelve chlorinated hydrocarbons. Because six ions are monitored for each component, the suspected component and its level should be made known to the analyst to minimize time spent on the confirmatory procedure. A list of the monitored ions and approximate retention times is given in Table 1.

5.004B. Confirmatory Criteria

The method has not been validated: GC/MS data has been obtained for samples prepared by only one analyst, and only one mass spectrometer. Thus only tentative confirmatory criteria have been established:

- (a) At least four of the six ions monitored for any chlorinated hydrocarbon must be present.
- (b) At least two ratios, relative to the most intense ion, must be within ± 15 percent of those found in samples spiked at the action level, or level found in the suspect sample.
- (c) At least one of the two ion ratios must involve ions from the highest m/z value isotopic cluster monitored, if ions in that cluster have a relative intensity greater than 15 percent.
- (d) The retention time must be within ± 5 percent of that found for fortified samples.

5.004C. Apparatus

GC Column: 30 M OV-1, narrow bore (Hewlett Packard *Ultrabond*, part 19091A, option 105), or equivalent.

Gas: He, 20 psi

Oven: Starting temperature at 180°C, held for 1 minute, then programmed at 250°C at 16°/min. The oven is held at 250°C for several minutes after the chlorinated hydrocarbon (CHC) of interest has eluted. The "solvent elution time" and "run time" are varied to create a small time window during which the CHC of interest elutes. This procedure, which is experimentally determined by the analyst/MS operator, minimizes the time during which the filament is on, thereby maximizing source cleanliness.

5.004D. Reagents

- (a) See Reagents sections of 5.001, 5.002, or 5.003
- (b) Dry nitrogen
- (c) Hexane - Burdick and Jackson, distilled in glass, or equivalent

5.004E. Determination

- (a) Carefully evaporate to dryness the sample extract prepared for GC injection by the acetonitrile partition/Florisil column cleanup procedure (5.001), the micro alumina column screening procedure (5.002), or the gel permeation chromatography method (5.003).

(b) Dilute to 100-200 ul with hexane. The volume may be varied such that injection of no more than 5 ul into the GC/MS produces a sufficient number of counts at each monitored ion so that confirmatory criteria can be met, using samples fortified at or below 80 percent of the action level.

5.004 Table 1—MONITORED IONS

Compound	Approximate Retention Time	Approximate Relative Ion Intensities					
BHC	4.8	<u>217</u> 100	<u>219</u> 95	<u>181</u> 97	<u>145</u> 21	<u>183</u> 100	<u>111</u> ?
HCB	4.9	<u>284</u> 100	<u>286</u> 88	<u>249</u> 24	<u>142</u> 31	<u>214</u> 20	<u>107</u> ?
Lindane	5.1	<u>183</u> 100	<u>219</u> 92	<u>217</u> 83	<u>181</u> 86	<u>146</u> 20	<u>111</u> 62
Heptachlor	6.2	<u>272</u> 100	<u>270</u> 59	<u>237</u> 23	<u>372</u> 13	<u>374</u> 13	<u>337</u> 34
Heptachlor Epoxide	7.2	<u>355</u> 100	<u>353</u> 80	<u>237</u> 20	<u>263</u> 13	<u>253</u> 21	<u>272</u> ?
Oxychlordan	7.3	<u>115</u> 100	<u>387</u> 69	<u>389</u> 74	<u>310</u> 34	<u>312</u> 26	<u>424</u> 11
DDE	8.2	<u>318</u> 100	<u>316</u> 76	<u>248</u> 42	<u>246</u> 92	<u>210</u> ?	<u>176</u> 14
Dieldrin	8.5	<u>263</u> 100	<u>279</u> 52	<u>277</u> 98	<u>191</u> 37	<u>108</u> 69	<u>237</u> 26
Endrin	9.0	<u>345</u> 100	<u>347</u> 58	<u>263</u> 66	<u>245</u> 56	<u>248</u> 50	<u>173</u> 40
TDE	9.1	<u>235</u> 100	<u>237</u> 68	<u>212</u> 20	<u>178</u> 31	<u>176</u> 17	<u>165</u> 30
DDT*	—	—	—	—	—	—	—
Methoxychlor	12.1	<u>227</u> 100	<u>223</u> 43	<u>238</u> 40	<u>152</u> 37	<u>308</u> 18	<u>310</u> 12
Mirex	15.1	<u>272</u> 100	<u>274</u> 37	<u>270</u> 48	<u>237</u> 21	<u>235</u> 18	<u>143</u> ?

* Mixture of isomers. Relative ion intensities not determined.

5.005 Analysis and Confirmation for Ethylene Dibromide in Animal Tissue by Codistillation

5.005A. Theory

Ethylene dibromide (EDB) has very low solubility in water and has a high vapor pressure, making codistillation over water with another solvent (hexane), a practical separation technique. The hexane - EDB condensate is trapped in a Barrett distilling receiver that is cooled in an ice bath. The water is drained from the receiver, the volume of hexane read, and the hexane layer is transferred to a 20 ml scintillation vial containing 2-3g Na_2SO_4 . The dried condensate is injected directly on a gas chromatograph equipped with an electron capture detector.

5.005B. Apparatus

- (a) Gas Chromatograph Hewlett-Packard 5880 (or equivalent) equipped with Electron Capture detector (^{63}Ni)
- (b) 6 ft. \times 2 mm glass column packed with 15% OV-17 on 80/100 mesh Chromosorb WAW or 20% OV-225/20% OV-17 (2 + 1) on 80/100 mesh Chromosorb W-HP
- (c) Argon/Methane carrier gas 95/5: 20 ml/min for 15% OV-17 column, 37 ml/min for 20% OV-225/20% OV-17 column
- (d) Oven temperature programmed from 100°C for 8 min at 16°C/min to 220°C, hold for 8 to 10 min, time delay 15 min for equilibration. Injection temperature 160°C, detector temperature 350°C. (See Notes 1 and 5).
- (e) Scintillation vial, 20 ml, Wheaton Scientific No. 98654, or equivalent (See Note 2)
- (f) Volumetric flasks 100 ml and 10 ml
- (g) Syringes 10 μl , 25 μl , 100 μl , and 1000 μl
- (h) Condenser water jacket, Kimax No. 18190 with 24/40 ground glass fitting, or equivalent
- (i) Barrett distillation receiver, 20 ml, Pyrex No. 3622, or equivalent
- (j) 1000 ml round bottom flask with 24/40 ground glass fitting single neck (See Note 3)
- (k) Heating mantle, 1 liter size, Glas-Cal Apparatus Company, 711 Hulman Street, Terre Haute, IN, 47803, Catalog No. 0-408, or equivalent (See Note 3)

5.005C Reagents

- (a) 1,2 - dibromoethane (>99%), ALDRICH GOLD LABEL No. 24.0065-6, b.p. 131-132°C
- (b) Hexane, UV grade, Burdick and Jackson, or equivalent
- (c) Distilled water, Waters Milli Q treated, or equivalent
- (d) Sodium Sulfate, reagent grade (tested for interfering peaks) anhydrous crystals

5.005D Sample Preparation

To prevent cross contamination sample is finely chopped on filter paper just before analysis.

5.005E Determination

- (a) Weigh 10 grams of finely chopped sample into 1000 ml round bottom flask, add 300 ml distilled water, 10 ml hexane, and boiling chips.

- (b) Connect to distillation apparatus and place in heating mantle
- (c) Increase heat until water just boils and collect distillate until 2-3 ml water layer appears in Barrett trap.
- (d) Drain water layer and discard.
- (e) Add contents of trap (8.8 to 10.0 ml hexane) to 20 ml scintillation vial containing 2-3 grams of anhydrous Na_2SO_4 , shake vial and let stand. When hexane is no longer cloudy, inject 5 μl on G.C.
- (f) Prepare a reagent blank by distilling 10 ml hexane with 300 ml water and collect in the same manner as a sample.
- (g) Prepare a tissue blank by distilling 10 grams of the tissue of interest with 10 ml hexane and 300 ml water, and collect in the same manner as a sample.
- (h) Perform recoveries at 1 ppb equivalent EDB by repeating steps 5.005E. (a)-(e), above. Repeat with 100 ppb EDB equivalent, if samples are found in this range. Use standards 5.005F. (d) (i and ii), below.

Note (1) Detector temperature can be maintained at 260°C for greater sensitivity during the analysis but must be elevated at 350°C periodically to prevent contamination.

Note (2) Adhesive in foil lined cap of vial may produce interfering peaks on G.C. this can be prevented by adding a teflon liner to the cap.

Note (3) Flask size can be reduced to 500 ml and a hot water bath can be substituted for the heating mantle, if these items are not available.

Note (4) This method is also suitable for whole grains, using 10 grams of grain and 10 ml of hexane.

Note (5) Instrument should be adjusted to give approximately 50% full scale response and a retention time of about 5-6 minutes for a 1 ppb external standard.

5.005F Standards

- (a) Stock Standard (0.5 mg/ml) - add 23 μl of 2.179 specific gravity EDB to a 100 ml vol flask and dilute to volume with hexane.
- (b) Working Standard (0.5 $\mu\text{g}/\text{ml}$) - Add 100 μl of stock standard to a 100 ml vol. flask and dilute to volume with hexane.
- (c) External Standard (1.0 ng/ml) - Add 200 μl working standard to 100 ml vol flask and dilute to volume with hexane.
- (d) Recovery Standard

1 ppb - Add 1 ml working standard to 500 ml Hexane.

Add 10 ml recovery standard solution to 10g blank tissue.

5.005G Calculations

Each chromatogram should contain injections of hexane, reagent blank, tissue blank, external standard, (1ng/ml), and 1 ppb recovery standard. Calculation is made against the external standard without correction for recovery.

5.005H Confirmation of EDB Residue

- (a) Inject 5 μl of hexane to determine if there are any interfering peaks, prior to injecting the samples.

(b) For EDB residue between 1 and 5 ppb, inject 5 μ l of the 10 ml hexane distillate into the mass spectrometer utilizing the following equipment and conditions:

Gas chromatograph equipped with a 25 M OV-1 column. Injection port 150°C, column temperature 50°C. Use a splitless injection system for the analysis.

Operate mass spectrometer in the negative chemical ionization mode using methane as reagent gas. Tune the instrument on ion 633. Adjust the electronics for maximum response while just attaining separation between 633 and 634. Monitor ions 79 and 81. Interface temperature 200°C, Source temperature 200°C.

Minimum detectable amount should be between 1 and 5 μ g of EDB when monitoring ions 79 and 81, with a retention time of 4.5 minutes.

(c) For EDB residues between 5 and 20 ppb, concentrate the 10 ml hexane distillate to 1 ml at room temperature under a stream of nitrogen. Inject 5 μ l of this concentrate into the mass spectrometer using the same GC conditions as above (b) and the following MS conditions:

Operate mass spectrometer in the electron impact mode. Perform a normal tune procedure using PFTBA as standard. Source and interface remain at 200°C. Monitor ions 107 and 109.

Minimum detectable amount should be less than the 250 μ g injected.

A positive confirmation is reported if in procedure (b) both ions, 79 and 81, are present at the correct retention time and the ratio of 79 and 81 is approximately $1.00 \pm 10\%$, and in procedure (c) if both ions, 107 and 109 are present at the correct retention time and the ratio of 107 to 109 is approximately $1.10 \pm 10\%$.

Reference

Rains, D. and Holder, J., J. Assoc. Off. Anal. Chem., Vol. 64, pp 1252-1254, (1981)

5.006 Determination of Organophosphate Pesticide Residues in Liver and Muscle Tissue

5.006A. Theory

Fifty grams of liver or muscle tissue are weighed into a centrifuge bottle. The weighed sample is fortified with ethyl parathion as an internal standard and blended. A recovery sample, fortified with the working standard solution, is analyzed in parallel. Tissue is blended, centrifuged, and filtered. The filtrate is extracted with an ethyl acetate/hexane mixture by blending and then centrifugation. The extract is concentrated, reconstituted to specific volume and partitioned with acetonitrile. Further cleanup is accomplished by a charcoal column. The eluant is concentrated for GC analysis using a flame photometric detector.

The following organophosphates are quantitatively analyzed by this procedure: Dioxathion, Diazinon, Methyl Parathion, Fenitrothion, Malathion, Ethyl Parathion, Ruelene, Gardona, Ethion, Trithion, and Coumaphos.

Other organophosphate pesticides such as Chlorpyrifos, Disulfoton, and Trichlorfon can be qualitatively and quantitatively recovered by this procedure, but GLC retention times may be overlapping. The organophosphate pesticides listed above are all resolved when instrumental parameters and methodology specifications as described below are followed.

5.006B. Apparatus

- (a) Centrifuge bottles, 250 ml, polypropylene, Screw Cap, Scientific Products #C4162-250, or equivalent
- (b) Tissuemizer, Tekmar Model STD, or equivalent
- (c) Centrifuge, Sorvall RC 5, or equivalent
- (d) Round bottom flask, 500 ml, Fisher Scientific #10-067G, or equivalent
- (e) Filter paper, Whatman, 2 V fluted, 18-24 cm, or equivalent
- (f) Rotary Evaporator, Buchler Flash, Evaporator, Buchler Instruments, or equivalent
- (g) Volumetric Flask: Class A 100 ml, 10 ml, 50 ml; Scientific products #F4665-100, #F4665-10A, #F4665-50, or equivalents
- (h) Screw Cap scintillation vials, Curtin Matheson Scientific #250-332, or equivalent
- (i) Graduated concentrating tubes, 4 ml, 10 ml, Kontes #570050-0425, #570050-1025, 15 ml, Curtin Matheson #053-132 or equivalents
- (j) Pipet: 1 ml, 10 ml, Class A, Fisher Scientific #13-651A, #13-651K, or equivalents
- (k) N-Evap, Organomation Model 111 or 112, or equivalent
- (l) Vortex mixer, Fisher Scientific #12-814, 12-810, or equivalent
- (m) Disposable Pasteur Pipets, 9", Curtin Matheson #063172, or equivalent
- (n) Silanized glass wool, Applied Science #14502, or equivalent
- (o) Micropipet, 0.1 ml, Fisher Scientific #21-164-2H, or equivalent
- (p) Syringes, 5 ml, Curtin Matheson Scientific, Glass with Luer-Lok Tip #221-465, Needle (25 gauge) #221-986, and 10 ul Hamilton #801, or equivalents

(q) Gas Chromatograph, Tracor Model #222, or equivalent, equipped with:

(1) Phosphorus/Sulfur, dual flame photometric detector (Sulfur detector is optional), with the following instrumental conditions:

Nitrogen Carrier Gass	80 ml/min
Hydrogen	150 ml/min
Air	60 ml/min*
Oxygen	20 ml/min

NOTE: Set gas flow rates according to manufacturer's instructions if GC is other than model specified above.

Temp. Program	160°C hold 2 min 160°C 250°C @ 8°C/min 250°C hold 6-0 min
Injection Temp.	220°C
Flame photometric Detector Temp.	190°C (should be 150°-160°C before flame is lit).

(2) Nitrogen-Phosphorous flame Ionization Detector with the following instrumental conditions:

Helium carrier gas	40 ml/min
Hydrogen	3 ml/min
Air	80 ml/min*
Temp. Program	160°C hold 1 min 160°C-250°C @ 8°C/min 250°C hold 17-18 min
Injection Temp.	225°C
Nitrogen-Phosphorus FID Temp.	300°C

* The air and or O₂ to H₂ ratio can be critical for some organophosphate compounds. The air flow should be adjusted to produce the best response without increasing baseline noise.

(r) Column: 6 ft × 4 mm I.D. column, 10% OV-101 on Chromosorb W - High performance, (80/100 mesh) condition column at 275-280°C overnight @ 50 ml/min nitrogen flow

(1) Carbowax 20 M Column Treatment (Fig. 1):

The conditioned column is treated with Carbowax 20 M in the following manner: About 2" (5 cm) of support material coated with 10% Carbowax 20 M is placed in the injection port area of the column and separated from the support in the packed column with a small amount of silanized glass wool. After installing the column in the instrument, the injection port temperature is raised to 230°C and the column temperature to 225°C. These conditions are maintained overnight with a flow of 10-20 ml/min of nitrogen. After treatment, the column is removed from the instrument and the 2" of carbowax material is removed and discarded. The column is reinstalled for analyses. The internal standard (Ethyl Parathion) should have a retention time of ca 9 min with a ca 40 percent full scale deflection for a 4 ul injection at the concentration described in Table 1.

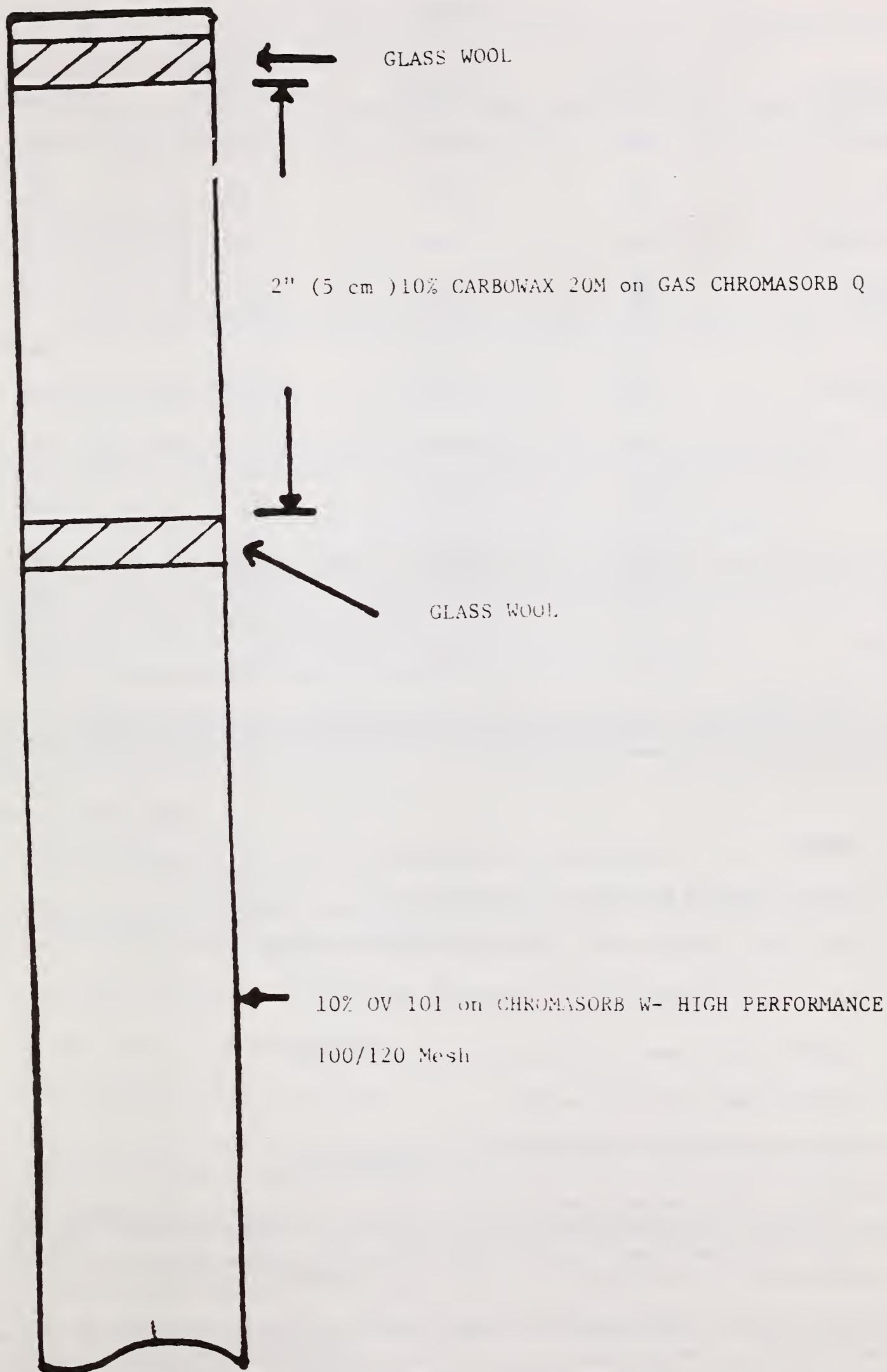


Figure 1.—Carbowax Conditioning of GC Column for Organophosphate Residue Analysis

TABLE 1

Organophosphate	Stock Solution Concentration	Working Std. Concentration	GC Standard Concentration	Concentration in 4 ul Injection
Dioxathion	1.0	54.00	2.70	10.80
Diazinon	0.8	15.98	0.799	3.20
Methyl Parathion	1.0	19.30	0.965	3.86
Fenithrothion	0.8	16.23	0.812	3.24
Malathion	1.0	20.23	1.01	4.04
Ethyl Parathion	0.8	15.94*	0.797	3.18
Ruelene	1.0	29.35	1.47	5.88
Gardona	1.0	51.11	2.56	10.24
Ethion	0.8	15.70	0.785	3.14
Trithion	0.8	24.11	1.21	4.84
Coumaphos	1.0	77.62	3.88	15.52

* Used as an internal standard

NOTE: The concentrations of the organophosphates are approximate. The analyst should adjust the concentrations, if needed, to get an optimum response and sensitivity for the instrument and detector used.

5.006C. Reagents

- (a) Ethyl acetate (EtAOc), Pesticide quality, (Burdick and Jackson, or equivalent)
- (b) Petroleum ether, pesticide quality, (Burdick and Jackson, or equivalent)
- (c) Hexane, pesticide quality, (Burdick and Jackson, or equivalent)
- (d) Acetonitrile, pesticide quality, (Burdick and Jackson, or equivalent)
- (e) Acetonitrile saturated with petroleum ether
- (f) Methylene Chloride (CH_2Cl_2), (Burdick and Jackson, or equivalent)

(g) Celite 545 - Johns-Manville, Denver, CO 80217. Must be free of substances which interfere with GLC determinations. Remove electron capturing substances by slurring with 1 + 1 hydrochloric acid- H_2O while heating on steambath. Wash with H_2O until neutral. Wash with Methanol, then EtOAc and then with hexane, and dry. Remove substances which interfere with phosphorus-selective GLC by heating at 600°C in a muffle furnace for at least 4 hours.

(h) Charcoal - Nuchar C-190N available from Eastman Kodak or Scientific Products #5790-500. To cleanup charcoal sufficiently for use in charcoal column cleanup, slurry 200 g with 500 ml conc. HCl, cover with watch glass, and stir magnetically while boiling 1 hr. Add 500 ml H_2O , stir, and boil additional 30 min. Collect charcoal in Buchner funnel and wash with H_2O until washings are neutral to universal indicator paper. Dry at 130°C in a forced-draft oven overnight.

(i) Magnesium oxide - Sea Sorb 43 (Westvaco #2641) (Fisher Scientific Co., Cat. #S-120). Treat as follows: Slurry about 500 g with distilled H_2O , heat on steambath about 30 min., and filter with suction. Dry overnight at 105° — $130^{\circ}C$ and pulverize to pass #60 seive. Store in closed jar.

(j) Charcoal absorbent mixture - Mix 1 part acid-treated charcoal, 2 parts hydrated magnesium oxide, and 4 parts Celite 545, acid-washed. Keep in a closed container. For example: 100 grams charcoal, 200 g hydrated MgO , 400 grams Celite 545.

(k) Standard Solutions:

(1) Primary Standards:

Can be obtained from EPA, Reference Standards Repository (MD-8), Research Triangle Park, North Carolina 27711

(2) Standard Stock Solutions:

Weigh 40-50 mg of standard into a screw cap scintillation vial (or other closable container). Dissolve with a small volume of Ethyl Acetate. Transfer to a 50 ml volumetric flask and dilute to volume. Store stock solutions in freezer.

(3) Working Standard Solution (used as a fortification solution for recovery sample):

Determine the number of milliliters of each Stock Solution needed to approximate the working standard concentrations in Table 1. Combine the appropriate amounts of each standard in a 100 ml volumetric flask and dilute to volume with Ethyl Acetate. Keep refrigerated when not in use.

(4) Gas Chromatography Standard (for injection):

Dilute 0.5 ml of Working Standard Solution to 10 ml with Ethyl Acetate. Keep refrigerated when not in use. Prepare fresh solution about every two weeks.

5.006D. Determination

(a) Weigh 50 grams of liver or muscle into 250 ml plastic centrifuge bottle.

(b) Spike samples with 1 ml of internal standard ethyl parathion. Spike recovery with 1 ml of recovery spiking solution. (Ethyl parathion alone, at working standard concentration).

(c) Add 100 ml of a mixture 70% EtOAc and 30% hexane, V/V, to each sample.

(d) Blend sample for 1 minute (tissuemizer).

(e) Centrifuge for 10 minutes at 2,000 rpm.

(f) Filter extract into a 500 ml round bottom flask. (2 V fluted filter paper 18 cm or 24 cm.) If blood has separated during centrifugation, do not let blood get into filter.

(g) Add 100 ml of 70% EtOAc - 30% hexane to tissue and again tissuemize for one minute.

(h) Centrifuge at 2,000 rpm for 10 minutes.

(i) Filter extracts into same 500 ml round bottom in step (f) (combine the two extracts). If blood has separated during centrifugation, do not let blood get into filter.

(j) Evaporate extract to 90-95 ml on roto-evaporator (50° - $60^{\circ}C$).

(k) Transfer and dilute to volume with 70% EtOAc - 30% hexane mixture in 100 ml volumetric flask while rinsing round bottom flask into volumetric. Stopper and mix well.

(l) Transfer extract to plastic bottles with screw caps or 250 ml centrifuge bottles.

(m) Pipet 10 ml extract into graduated concentrator tube (15 cc). Place plastic bottles in freezer.

(n) Reduce volume to approximately ½ ml on N-Evap.

(o) Bring volume to 3 ml with petroleum ether. Mix well. (Use an additional 1-2 ml of petroleum ether for samples with excessive fat.)

(p) Add 7 ml of acetonitrile (saturated with petroleum ether). Stopper and mix gently with a back and forth motion. Leave standing a few minutes to allow the layers to separate.

(q) Aspirate and discard top layer (petroleum ether layer). This should eliminate some of the fat.

(r) Add 2 ml of petroleum ether and repeat shakeout. Let separate. Aspirate and discard top layer.

(s) Concentrate on N-Evap. When volume reaches 2-3 ml, rinse sides with EtOAc. Continue to concentrate until volume is less than one ml.

(t) Make volume to one ml with EtOAc. Stopper tubes and mix well on vortex mixer.

(u) Mix charcoal adsorbent mixture well before each use. Weigh 0.3-0.4 gm of charcoal adsorbent mixture (Reagent j) for each sample. Place a small plug of silane treated glass wool in a 9" disposable Pasteur pipet. Add the adsorbent mixture to the pipet (column). Tap gently to settle. Add a small plug of glass wool on top of the adsorbent bed to prevent disruption while adding solvent to the column.

(v) Add 20 ml of eluting solvent (CH_2Cl_2) to a flask and place next to the sample flask. (Label flasks.)

(w) Pre-rinse columns with 3 ml CH_2Cl_2 from the flask containing 20 ml MeCl_2 . Let drain and discard CH_2Cl_2 eluate.

(x) Collect extract from column in an Erlenmeyer flask. Place flasks under each tube to collect eluant. (Label flasks!)

(y) Add 0.1 ml of the sample to column via disposable micropipet. Rinse pipet with 1 ml eluting solvent onto the column using a syringe.

(z) Let drain to near top of column packing. Add eluting solvent to column. *Do not let columns go dry.* Add eluting solvent until the remainder of the 20 ml have been used. Approximately 30-45 minutes required.

(aa) Concentrate on N-Evap. Transfer extract to concentrator tube. When volume reaches 1-2 ml, rinse empty flask with 2-3 ml of ethyl acetate and add to evaporating tube, stopper and mix. Continue evaporation.

(bb) Concentrate to 0.1 ml (do not evaporate to dryness). For FPD, make volume to 0.2 ml with EtOAc. Mix samples on vortex mixer. Stopper each tube. For FID, add 3-4 ml hexane, reconcentrate to 0.1 ml (do not evaporate to dryness). Make volume to 0.2 ml with hexane.

NOTE: A larger dilution may be made for use with automatic injectors.

(cc) Inject appropriate volume of sample solution into the gas chromatograph using the conditions described in 5.006 B. (q), above.

NOTE: Before injection of samples, it is advisable to "condition" the column packing by injecting a high concentration standard followed by 3-4 injections of the gas chromatography standard. This procedure reduces chances of adsorption of compounds at the low levels (especially polar compounds).

5.006E. Calculations

Concentration of an organophosphate (OP) present is calculated using either peak height or peak area as follows:

$$(\text{OP}) \text{ ppm} = \frac{\text{ul std inj}}{\text{ul sample inj}} \times \frac{\text{pk ht (area) sample}}{\text{pk ht (area) std}} \times \frac{\text{Conc std ug/ml}}{2.5 \text{ gm/ml}}$$

5.006F. Alternate or Confirmation Columns

- (1) 4% SE-30/6% OV-210 coated on Gas Chromosorb Q or Chromosorb W-High Performance 80/100 mesh
- (2) 5% or 10% OV-210 coated on Gas Chromosorb Q or Chromosorb W-High Performance 100/120 mesh
- (3) 1.5% OV-17/1.95% OV-210 coated on Gas Chromosorb Q or Chromosorb W-High Performance 100/120 mesh

Tables of elution patterns for these 3 columns can be found in *Manual of Analytical Methods for the Analysis of pesticide Residues in Human and Environmental Samples* - U.S. Environmental Protection Agency.

(4) 2% stabilized DEGS on Chromosorb W-Acid Washed 80/100 mesh. This column separates parent compounds from the oxygen-analogs. Also effects elution order of many of the organophosphates. *Pesticide Analytical Manual - Volume I, Table 334-A* - Food and Drug Administration.

References:

A General Method for Organophosphorous Pesticide Residues in Non-fatty foods:

Storherr, R. W., Ott, P., and Watts, R. R., JAOAC 54, 513-516 (1971)

Charcoal Column Cleanup Methods for Many Organophosphorous Pesticide Residues in Crop Extracts:

Watts, R. R., Storherr, R. W. and Pardue, J. R. JAOAC 52, 522-526 (1969)

GLC Retention Times of Pesticides and Metabolites Containing Phosphorous and Sulfur on four Thermally Stable Columns:

Bowman, M.C. and Beroza, M., JAOAC 53, 499-508 (1970)

Gas-Liquid Chromatographic Column Preparation for Adsorptive Compounds:

Ives, N. Fred. and Guiffrida, Laura. JAOAC 53, 973-977 (1970)

Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples: U.S. Environmental Protection Agency Sections 4, B(1)-B(5)

Pesticide Analytical Manual - Volume I - Food and Drug Administration, Sections 230.00-232.45, Table 334-A

5.007 Determination of Mercury in Liver, Muscle, Kidney or Hair by Atomic Absorption Spectrophotometry

5.007A. Theory

The sample is digested with a sulfuric acid-potassium permanganate solution to free all organic mercury compounds as ionic mercury. Excess permanganate is destroyed with hydroxylamine hydrochloride, and further reduction with stannous chloride to metallic mercury makes possible the measurement of the mercury vapor in the air. The lower limit of detection is 0.01 $\mu\text{g Hg}$.

5.007B. Apparatus

(a) Atomic absorption spectrophotometric equipment capable of continuum background correction is employed in the analysis. A mercury electrodeless discharge lamp (EDL) is used as a resonance source. With the EDL, background correction is difficult to achieve; therefore, a trap of concentrated sulfuric acid is used. (See Reference 3)

(b) Reaction flasks, pear shaped, 100 ml, 14/20 standard taper (Kontes Glass Company, Vineland, NJ 08360, K-294250 or equivalent).

(c) Continuous flow spectrophotometric cell, cylindrical, 100-150 mm, with tube on each end with push backs. (Optical Cell Company, Inc., 10792 Tucker Street, Beltsville, MD 20705—No. 4-435 or equivalent).

(d) The aeration equipment is illustrated in Figure 1. If continuum background correction is unavailable, concentrated sulfuric acid can be put in the bottom of the water trap to within 5 mm of the end of the tube. Any number of compounds, such as hydrocarbons of water, display molecular absorption in this region.

(e) Aeration tube should have an extra coarse frit (Kontes Glass Co. Sketch No. 001603-21, Quotation No. 37866, or equivalent).

(f) Water trap (Kontes Glass Co.—Sketch No. 001602-21, Quotation No. 37866, or equivalent).

(g) Modified 3-way stopcock: Figure 2 shows a full sized view. (Kontes Glass Co.—Quotation No. 38106), or equivalent.

(h) Flowmeter: Should have a range of 0-1.7 liters/min.—Brooks Sho-Rate with a R-2-15A tube using a steel ball (A.H. Thomas Company, Post Office Box 779, Philadelphia PA 19105, 5545-B (case) and 5545 (tube)), or equivalent.

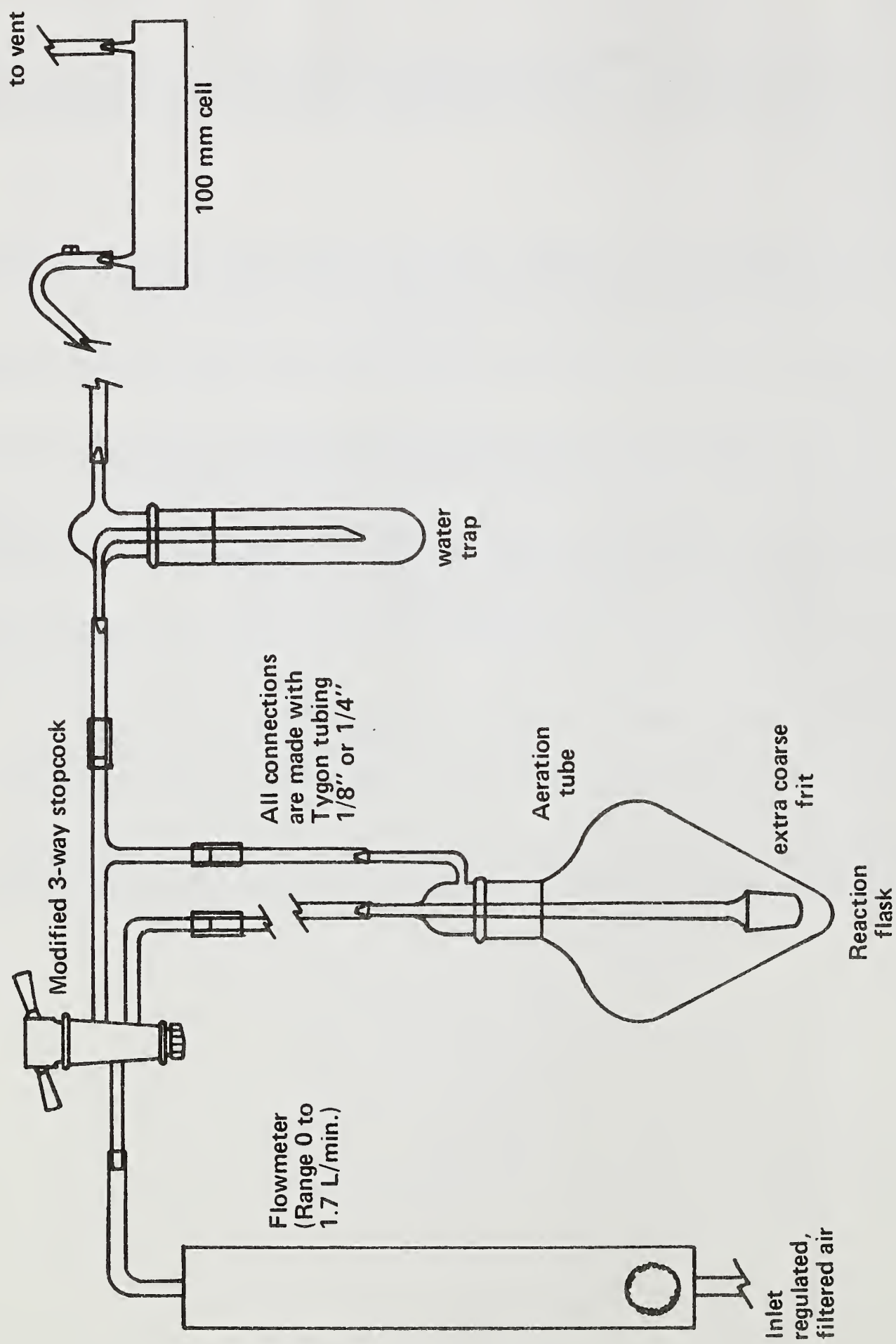


Figure 1.—Flow Diagram For Mercury Analysis

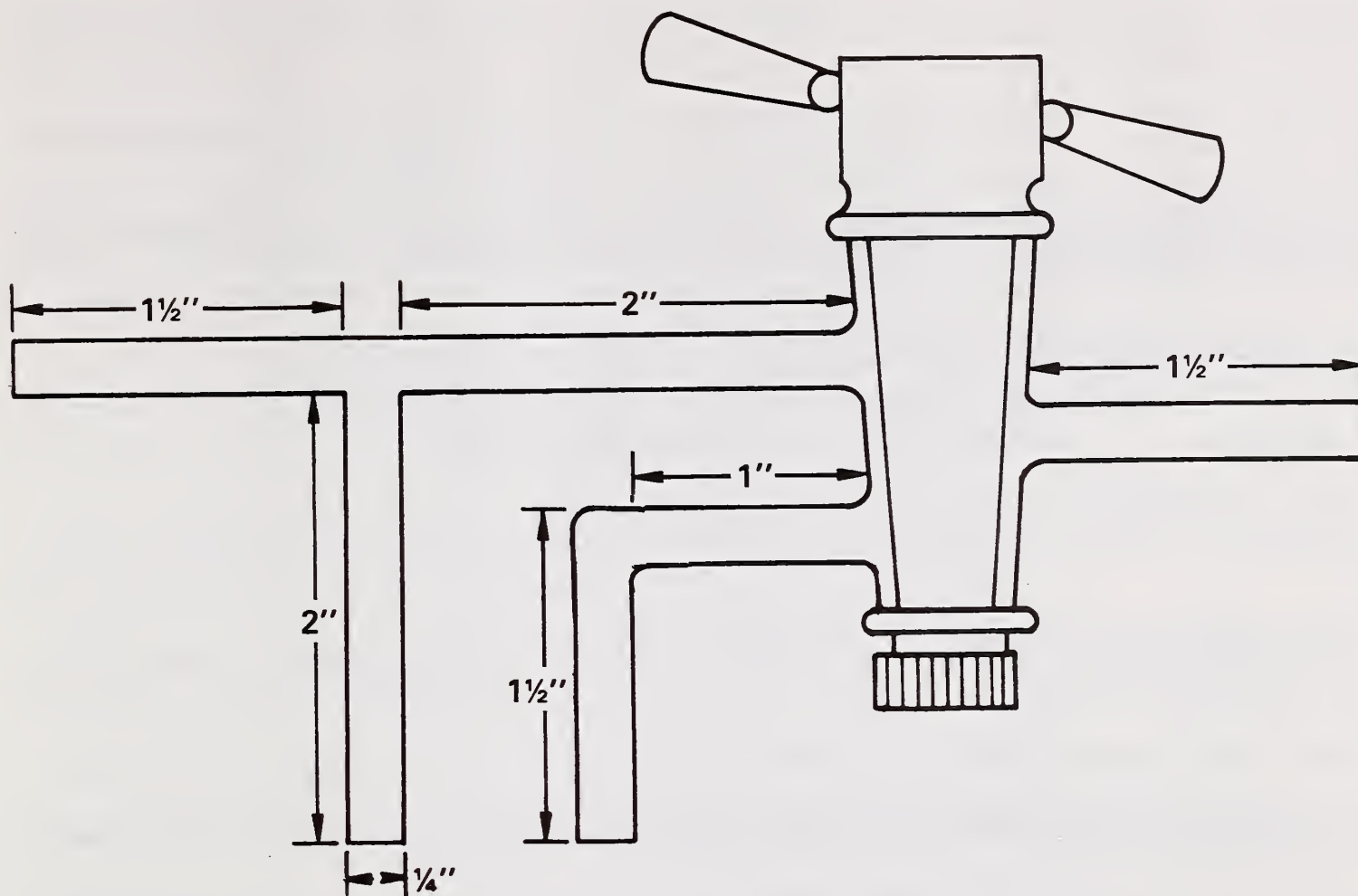


Figure 2.—Modified 3-Way Stopcock

5.007C. Reagents

- (a) Concentrated, Reagent Grade Nitric Acid, 1 + 1 with water (v/v).
- (b) Hydroxylamine Hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$)[#] solution, 10 percent (w/v): Dissolve 25 g of reagent grade $\text{NH}_2\text{OH}\cdot\text{HCl}$ in about 200 ml of distilled water. Transfer to a 250 ml volumetric flask, dilute to volume with distilled water and mix well.
- (c) Potassium Permanganate (KMnO_4)[#] solution, 6 percent (w/v): Completely dissolve 60 g of reagent grade KMnO_4 in about 800 ml of distilled water in a 1 liter beaker using a heated magnetic stirrer. Transfer the solution quantitatively to a 1 liter volumetric flask, cool, dilute to volume with distilled water and mix well.
- (d) Stannous Chloride (SnCl_2)[#] solution, 10 percent (w/v). (Prepare fresh every week.

Dissolve 20 g of reagent grade $\text{SnCl}_2\cdot 2\text{H}_2\text{O}$ [#] in 40 ml of warm concentrated hydrochloric acid. When all the stannous chloride has dissolved, add 160 ml of distilled water. Mix well and store in a 250 ml reagent bottle.
- (e) Sulfuric Acid (H_2SO_4)[#] concentrated: Reagent grade.

[#]Before committing samples, check different suppliers and check each lot of these compounds. The amount of mercury found in them varies markedly.

(f) Inorganic Mercury Standard, 1000 $\mu\text{g/ml}$. (Fisher Scientific Co., 711 Forbes Ave., Pittsburg, Pa. 15219; catalog No. SO-M-114 or equivalent).

(g) Mercury Standard Solution: 10 $\mu\text{g Hg/ml}$ (Note: 100 $\mu\text{g/ml}$ standard may be prepared as an intermediate step if desired.)

Pipet 1.0 ml of reagent (f) into a 100 ml volumetric flask containing 2 ml of redistilled nitric acid. Dilute to volume with distilled water and mix well.

(h) Preparation of standard curve: Make fresh daily.

ml reagent (g)	Final volume with Distilled water	μg Mercury in 20 μl
1	10 ml	0.02
3	10 ml	0.06
5	10 ml	0.10

(i) Organic Mercury Standard, 1000 $\mu\text{g/ml}$: (Alfa Products, P.O. Box 299, 152 Andorf Street, Danvers, Ma. 01923; catalog No. 88036, or equivalent).

(j) Methyl Mercury Standard Solution: 10 $\mu\text{g/ml}$.

Pipet 1.0 ml reagent (i) into a 100 ml volumetric flask, add 2.0 ml redistilled concentrated nitric acid and dilute to volume with distilled water.

(k) 5.0 $\mu\text{g/ml}$ Fortification Standard:

Pipet 5.0 ml of reagent (j) into a 10 ml volumetric flask and dilute to volume with distilled water.

Note: If samples are encountered with higher amounts of Hg, the standard curve (step 5.007C(h)) may be extended using 0.2, 0.6 and 1.0 $\mu\text{g Hg}$ in 20 μl standard solutions.

5.007D. Determination

5.007D1. Sample Preparation

(a) Muscle tissue—Eliminate as much fat from tissue as possible, pass rapidly three times through food chopper with plate opening less than or equal to $\frac{1}{8}$ inch, mixing thoroughly after each grinding.

(b) Liver or Kidney—Eliminate as much fat and connective tissue as possible from both the kidney and liver. Place tissues in a separate blender jar and blend until well homogenized (Waring Blendor should be used with extreme care when blending). Blend 1 minute; permit blender to cool for not less than 1 minute before blending again. Do not use variable transformers to control speed of blender. Freeze tissues until determinations are ready to be run on each.

(c) Hair—Wash with plain tap water to remove extraneous material, rinse with distilled water, and then dry. Place in freezer until ready to start determinations.

5.007D2. Digestion

(a) Clean all glassware with reagent (a) and rinse with distilled water just before *each* use.

Note: Avoid use of towels which may contain mercury, and do not use detergents.

(b) Place approximately 0.600-0.750 of the homogenized tissue (muscle, liver or kidney), or 0.5 g hair, into a tared 100 ml pear-shaped flask, taking care that all of the sample is deposited in the bottom of the flask and none is left in the

neck. Reweigh the flask and obtain the sample weight to the nearest 0.01 g, by difference. Cap the flask with a clean 10 ml beaker inverted over the top of the flask. This beaker is left on the flask during all stages of the digestion procedure.

(c) Pipet 5.0 ml of concentrated H_2SO_4 into the flask and place it on a steam bath to digest the sample (20-45 minutes is usually sufficient). Swirl flask during digestion to break up particles. The completed digested sample will form a highly colored solution with no pieces of undissolved matter, although the solution may be slightly cloudy. *NOTE:* Great care must be exercised at this point that all the sample is actually in solution and that there are no particles on the sides of the flasks or suspended in the solution. If this is not done the digestion will not be complete.

(d) When the sample is digested, place the flask into an ice bath for 5-10 minutes. Then pipet 15.0 ml of 6 percent KMnO_4 solution (CAUTION); into the flask, Swirl the flask, gently at first and then vigorously, until sample is well mixed. Place sample in rack and continue until KMnO_4 has been added to all samples.

(e) Swirl and place the flask on a steam bath and allow the sample to digest further. Swirl the flask occasionally, and continue to heat until frothing ceases and all foam disappears (usually 15-20 minutes). Do not heat longer than is needed. Some foam may be present when reaction has stopped.

(f) Remove the flask from the steam bath and pipet a further 5.0 ml of 6 percent KMnO_4 (10.0 ml of 6 percent KMnO_4 for hair samples) solution into it.

(g) Place the flask back on the steam bath for 15 minutes.

(h) Cool the flask to room temperature and analyze the contents for mercury by atomic absorption.

5.007D3. Recovery Standard for Checking Analytical Performance

Using a microliter syringe add 20 μl (0.10 μg) of reagent (k) to 0.75 g of homogenized tissue in 100 ml pear-shaped flask. Proceed as in steps (a)-(h) of the digestion procedure.

5.007E. Preparation of a Calibration Curve (To be carried out at least once per day)

(a) Into each of eight clean, 100 ml flasks. pipet 20 ml reagent (c). Cap the flasks with clean 10 ml beakers inverted over the tops of the flasks.

(b) Cool the flasks in an ice bath for a few minutes, then slowly and cautiously pipet 5.0 ml of concentrated sulfuric acid into each flask. Swirl gently and allow to cool.

(c) Using a microliter syringe, add 20 μl of each inorganic standard (1 μg , 3 μg and 5 $\mu\text{g}/\text{ml}$) so that duplicate standards are obtained for each level. The levels will be 0 (none added), 0.02 μg and 0.06 μg and 0.10 μg .

(d) Cool the flasks to room temperature prior to the aeration and atomic absorption analysis.

NOTE: Reagent blanks should show absorbances which are equivalent to 0.02 μg or less of Hg. If reagent blanks are higher, check glassware cleanliness and reagent solutions. Some checking of various suppliers' reagents may be necessary to determine those most suited to this analysis.

5.007F. Atomic Absorption Analysis

(a) Set up the atomic absorption equipment according to the diagram in Figure 1. Set the air flow to give good sensitivity and low foaming (0.7—1.0 liters/min.).

(b) Add 5.0 ml of 10 percent $\text{NH}_2\text{OH}\cdot\text{HCl}$ (10.0 ml in the analysis of hair) solution to the digestion flask and swirl to dissolve the manganese oxides. Add about 10 ml of distilled water to bring the total volume to 40 ml \pm 2.0 ml.

NOTE: This solution should not have any color or any particles suspended in it, but it may be slightly cloudy. See *NOTE* under Digestion—(c).

(c) Add 2 ml of 10 percent $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution and immediately aerate the solution.

(d) Discontinue the aeration after the recorder pen has settled back to within a few (2 or 3) chart divisions of its original baseline—usually 1 to 1½ minutes depending on the actual aeration rate.

5.007G. Calculations

Calculate area of peak by measuring its height in mm and multiplying by the mm width at one half its height (read to the nearest 0.25mm.) Standard curve is calculated using linear regression.

The linear regression formula is: $Y = mX + b$

Where: $Y = \mu\text{g/g}$, $X = \text{area}$, $m = \text{slope}$, $b = \text{intercept}$

Calculate m and b . Calculate μg Mercury in sample and divide by sample weight in grams to obtain ppm.

References

(1) "Determination of Mercury in Fish (Atomic Absorption Spectrophotometric Method)", Method CAS-AM-70.10, June 11, 1970, Dow Chemical Company, Midland, Michigan 48640.

(2) Manning, D.C. "Compensation for Broad-Band Absorption Interference in the Flameless Atomic Absorption Determination of Mercury", Atomic Absorption Newsletter, Vol. 9, No. 5 (Sept.-Oct. 1970) pg 109.

(3) Kothandaraman, P., and Dallmeyer, J.F., "Improved Desiccator for Mercury Cold Vapor Technique", *Atomic Absorption Newsletter*, Vol. 15, No. 5, pp. 120-121, Sept.-Oct. 1976.

5.008 Arsenic Analysis (Spectrophotometric)

The sample is ashed at 600°C and dissolved in dilute hydrochloric acid. Zinc is added; the arsenic is distilled as arsine (AsH_3) and collected in an iodine solution. Ammonium molybdate is added to the solution forming a heteropoly molybdiarsenate, which upon the application of heat, is reduced by hydrazine sulfate to form the "molybdenum blue" complex. The developed color is read on a suitable spectrophotometer at 840 nm.

5.008A. Apparatus

- (a) Distillation apparatus—Kingsley-Schaffert distilling apparatus (Corning Glass Works, No. 33680) consisting of 125 ml flask, funnel trap, and bent dispersion tube.
- (b) Spectrophotometer suitable for reading at 840 nm.
- (c) Pipettes—various sizes.
- (d) Matched set of cuvettes approximately 19×105 mm.

5.008B. Reagents

Glassware should not be subjected to routine washing with soap or detergents which are often the source of phosphorus contamination. When soap or detergent is used, clean with aqua regia before use. Rinse delivery tubes by holding in slanted position with crook up and squirting jet of H_2O up and over inside crook until tube is filled; then rinse outside while tube drains. Repeat rinse three times. Rinse funnels in each direction alternately by filling end that is up and placing funnel on 1-hole rubber stopper in mouth of vacuum flask to pull H_2O thru frit by vacuum.

- (a) Tissue solvent: CHCl_3 (or benzene)-acetone-absolute alcohol (1 + 1 + 2).
- (b) Dilute hydrochloric acid: Dilute 372 ml conc HCl to 1000 ml with distilled water (4.5N).
- (c) Potassium iodide solution 15%: Keep in dark. Discard when solution turns yellow.
- (d) Stannous chloride solution: 40 percent in diluted HCl, (b). Store in contact with metallic tin.
- (e) Zinc: Shot of uniform size and shape, ca 0.5 g each. Prewash the zinc shots with petroleum ether to remove coating; remove solvent by evaporation on a steam bath and dry in an oven at 95°C.
- (f) Lead acetate solution: Prepare saturated aqueous $\text{Pb}(\text{OAc})_2 \cdot 3\text{H}_2\text{O}$ solution in dropping bottle. Prepare fresh weekly or when solution becomes cloudy.
- (g) Iodine solutions: (1) 0.02N. - Dissolve 8 g KI and 2.54 g I_2 in small amount H_2O and dilute to 1 liter with H_2O . Store in dark bottle. (2) 0.001N. - Dilute 5 ml 0.02N I_2 to 100 ml with H_2O . Prepare fresh daily.
- (h) Ammonium molybdate solution: Dissolve 7.0 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in warm mixer of 70 ml H_2SO_4 and 300 ml H_2O , cool, and dilute to 500 ml with H_2O .
- (i) Hydrazine sulfate solution: Dissolve 0.3 g $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{SO}_4$ in H_2O and dilute to 200 ml.
- (j) Absorbent Cotton—metal free.
- (k) Inorganic Arsenic standard solution. (19 $\mu\text{g}/\text{ml}$) - Dilute 10.0 ml of a 1000 μg As/ml solution (Fisher-Cat. No. SO-A-449, or equivalent) to 100 ml with Reagent (b).

(l) Organic Arsenic standard solutions. (Arsenilic acid-Fischer #1369, or equivalent).

1. 1 mg As/ml: Dissolve 0.2897 g arsenic acid (based on 100 percent purity) in water and dilute to 100 ml.
2. Dilute 1.0 ml of solution (ℓ) 1. to 100 ml with water. (10 µg As/ml).
3. Dilute 1.0 ml of solution (ℓ) 1. to 200 ml with water. (5 µg As/ml).
4. Dilute 1.0 ml of solution (ℓ) 1. to 500 ml with water. (2 µg As/ml).

NOTE: Because the form of arsenic used in raising animals and poultry is organic, the use of organic arsenic standards is preferable.

(m) Magnesium nitrate hexahydrate ($\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, reagent grade. -50 percent (W/V) solution — Dilute 500 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ to 1L with distilled water.

(n) Dilute nitric acid (50%): one volume HNO_3 and one volume of H_2O .

5.008C. Sample Preparation.

Note: A reagent blank must be analyzed with each set of samples, the absorbance of which should not exceed 0.15.

(a) Sample preparation may be varied according to the type of quantity of sample available. Where sample quantity is ample and a portion representative of the whole sample is desired, pass the entire sample through the fine plate of a meat grinder at least twice (liver will only make one pass, or it may be blended instead of ground).

(1) Mix sample thoroughly and weigh desired amount (usually 10g) into 50 ml Vycor crucible.

(2) Add 8 ml magnesium nitrate 50% solution (4 g magnesium nitrate hexahydrate per 10 g of sample); mix thoroughly with clean plastic stirring rod.

(3) Spread the mix in an even layer around the sides of the crucible.

(b) When samples are small, the tissue solvent may be employed to assist in comminuting and mixing.

(1) Weigh a convenient amount of the sample into an homogenizer or blender vessel, add the proper amount of $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (4 g per 10 g of sample) and add a convenient amount of the tissue solvent; usually 30ml per 10g sample.

(2) Blend about one minute and weigh into a Vycor crucible an amount that will contain the desired amount of sample.

(3) The excess solvent and water (from tissue) must be carefully evaporated before the sample is burned. Remove the solvent by placing the crucible on a steam bath: after all the solvent has been evaporated place the crucible in an oven at about 95°C to remove the water.

5.008D. Determination

(a) Place the crucible in a forced air oven. Dry at ca 90°C overnight or at ca 110°C for 4 hr. Place in cool muffle furnace which is set to gradually come to 600°C (this can be done overnight). When all visible carbon is burned, cool the crucibles and cool the furnace.

(b) Dampen the ash with 50% HNO_3 (do not dissolve ash completely.) Place on low heat hot plate to dry the ash.

(c) Replace the crucibles in the cooled furnace (100°C) and heat gradually to 600°C. Hold at 600°C about 1 hour or until it is certain that all nitric acid fumes have been driven off. (If the furnace is small, those crucibles near the door may

not be as hot as some others. In this case their positions should be reversed. When this is necessary, two periods of about 40 minutes each are necessary.)

- (d) Remove crucibles from the furnace and allow to cool.
- (e) Dissolve ash in 4.5 N hydrochloric acid. Three 10 ml portions of acid (delivered with an all glass syringe without needle) are used to dissolve and transfer the ash to the 125 ml flask and a fourth 10 ml portion is used to wash down the sides of the flask.
- (f) Cool.
- (g) Add 2.0 ml of 15 percent potassium iodide solution and mix thoroughly by swirling flask.
- (h) Add 1.0 ml of 40 percent stannous chloride solution and mix thoroughly by swirling flask. Allow to stand in ice bath at least 15 minutes (but not more than 30 minutes). The 15 minutes waiting time is most convenient for making other preparations for the distillation. Wet all glassware and allow to drain for 30 minutes.
- (i) Place 7.0 ml of 0.001N iodine in each cuvette.
- (j) Place a small ball of absorbent cotton in top of each funnel and dampen with saturated lead acetate solution. Using distilled water as a lubricant, join the delivery tube firmly to the funnel.
- (k) Fill a 600 ml beaker with finely crushed ice layered between the levels of the cuvette holder. Add ice water to about 2/3 the height of the beaker and place the cuvettes in the holder. (It may be necessary to make a path through the ice for the cuvette).
- (l) At the end of 15 minutes, lubricate the lower joint of the funnel with distilled water, add approximately 12.5 g of zinc (previously weighed) to the flask, join the flask and funnel firmly and place delivery tube in cuvette as quickly as possible. Allow distillation to continue 1 hour.
- (m) At the expiration of 1 hour, carefully and slowly remove the delivery tube from the cuvette allowing liquid to drain as tube is being removed.
- (n) Add 0.5 ml ammonium molybdate solution to the cuvette and mix thoroughly.
- (o) Add 0.3 ml hydrazine sulfate solution to the cuvette and mix thoroughly.
- (p) Place cuvette (in the cuvette holder) in a boiling water or steam bath for 10 minutes.
- (q) Remove from the bath, wipe the cuvette dry with soft, lintless material and place in cool, dark place for about 1 hour before reading.

5.008E. Preparation of Standard Curve

It is *necessary* that the standard curve be made from distilled arsenic and it is *advisable* that the added arsenic be carried through the entire procedure with an arsenic free sample of tissue.

Prepare standard curve with 10 g samples of arsenic-free liver and 8 ml of 50% of magnesium nitrate. When using the standard inorganic arsenic solution, 100 μg As/ml, add known amounts of arsenic with a 200 $\mu\ell$ syringe to each in a definite progression of 50, 100, 150, 200, and 250 $\mu\ell$, respectively equal to 5, 10, 20 and 25 run at one time. When using the standard organic (arsenilic acid) solutions, add known amounts of arsenic to each in a definite progression such as 0, 2, 4, 6, 8, 10, etc. micrograms of As or 0, 3, 6, 9, 12, 15, etc. micrograms of As. Repeat at least three times. Determine the mean for each level and prepare a fitted line by linear equation. The following suggested formula may be used:

$$Y = a + bX$$

The "X" values are the various levels of arsenic used (2, 4, 6, etc. micrograms) used in obtaining the data. The levels of "X" should be shown as micrograms per gram. The "Y" values are the means of the absorbance readings obtained at each level of arsenic. The "a" and "b" values are constants which are derived from the data.

Using the "X" and "Y" values to drive the "a" and "b" values and their subsequent use in the above equation is known as the "method of least squares."

A line plotted by this method will give the best fit for the data available. The "a" and "b" values are derived from the following equations:

$$b = \frac{\sum (XY) - \frac{\sum (X) \sum (Y)}{N}}{\sum (X^2) - \frac{\sum (X)^2}{N}}$$

$$a = \frac{\sum (Y)}{N} - \frac{b \sum (X)}{N}$$

N = the number of levels of X

Arsenic-free tissue (if not available, use previously analyzed tissue) with known amounts of standard arsenic added should be analyzed with actual samples to determine the efficiency of the system.

Calculations:

$$\text{Arsenic (ppm)} = A \cdot B$$

$$A = \frac{10g}{\text{Sample wt(g)}}$$

$$B = \text{microgram of arsenic per gram (standard curve)}$$

NOTE: 10g based on sample sized used to prepare standard curve.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

5.009 Arsenic Analysis in Meat and Meat Products by Atomic Absorption Spectrophotometry

This procedure utilizes the dry ashing technique used extensively by our laboratories with magnesium nitrate hexahydrate as an ashing aid. The procedure as written is applicable to relatively lean meat and meat by-products. Arsine is generated into an argon-hydrogen entrained air flame where quantitation is done by atomic absorption.

5.009A. Apparatus

- (a) Atomic absorption spectrophotometer capable of measuring the 1937A resonance line of arsenic and equipped with a recorder readout.
- (b) Three-slot burner head and hydrogen-argon-entrained air flame.
- (c) Check valve replacement for nebulizer in Figure 1. (Kontes No. K-931000 with 6mm capillary tubing, or equivalent.)
- (d) Erlenmeyer flasks, 125 ml standard taper 24/40 of uniform size and shape.

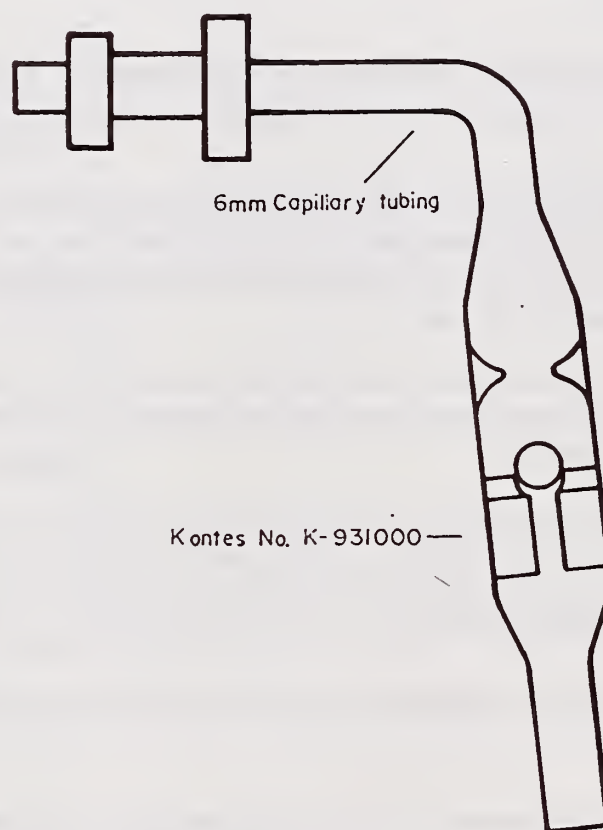


Figure 1

5.009B. Reagents

- (a) *Magnesium nitrate hexahydrate*: $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ crystals, reagent grade. (50% solution, W/V) - Dilute 500 g to one liter with distilled water.
- (b) *50 percent Nitric acid*: Prepare mixture of one part concentrated nitric acid and one part distilled water. Mix thoroughly.
- (c) *4.5 N hydrochloric acid*: To a 1000 volumetric add 372 ml of concentrated hydrochloric acid and bring to volume at room temperature.

(d) *Potassium iodide solution*: Prepare 15 percent solution daily one hour before use.

(e) *Sodium tetrahydride borate pellets (98%)*: Alfa stock No. 14122 (Ventron Corporation — P.O. Box 299; 152 Andover St., Danvers, Ma. 01923.)

(f) *1000 µg/ml Standard inorganic arsenic solution*: (Fisher Cat.No. SO-A-499, or equivalent.)

(g) *1000 µg/ml Standard organic arsenic solution*: (Arsenilic acid — Fisher Cat. No. 1369, or equivalent). Dissolve 0.2897 g arsenilic acid (based on 100 percent purity) in water and dilute to 100 ml.

(h) *100 µg/ml Arsenic solution*: Dilute 10 ml of the 100 µg/ml standard arsenic solution ((f) or (g)) to 100 ml with 4.5 N HCl.

(i) *Working Standards*: Dilute 1, 2, 3, 4, 5, 6, 7, 8 and 10 ml of Reagent (h) to 100 ml with 4.5 N HCL to give respectively, 1, 2, 3, 4, 5, 6, 7, 8 and 10 µg/ml standards.

NOTE: Because the form of arsenic used for raising animals and poultry is organic, the use of organic arsenic standards is preferable.

5.009C. Sample Preparation

(a) *Muscle tissue*—eliminate as much fat from tissue as possible, pass rapidly 3 times through food chopper with plate opening less than or equal to 1/8-inch, mixing thoroughly after each grinding.

(b) *Liver or kidney*—eliminate as much fat and connective tissue as possible from the kidney and/ or liver. Place tissue in a blender jar and blend until well homogenized. (Blender should be used with extreme care. Blend 1 min, permit blender to cool for not less than 1 min before blending again. Do not use variable transformers to control speed.) Freeze tissues until determinations are ready to be run.

(c) *Hair*—wash with plain tap water to remove extraneous material, rinse with distilled water, and then dry. Place in freezer until ready to start determinations.

5.009D. Ashing Procedure

(a) Weigh 5-15 g of ground sample into 50 ml Vycor crucible.

(b) Add 50% magnesium nitrate (2 g magnesium nitrate hexahydrate per 5 g of sample); mix thoroughly with clean plastic stirring rod.

(c) Spread the mix in an even layer around the sides of the crucible. The sample may now be placed in a cool furnace (below 80°C) or it may be dried in an oven at about 90°C overnight.

NOTE: Hair samples of up to 2 g may be analyzed. Into a 50 ml Vycor crucible weigh the hair sample to be analyzed, add 2 ml concentrated nitric acid and place on a steam bath to dissolve the hair, add 4 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and per-ash using an infrared lamp assembly similar to that shown in Figure 2.

(d) Ash the samples at 550° C bringing the furnace to temperature slowly (approximately 1 hr). Experience has shown that 6 hours reduces the carbon to an acceptable level. When all visible carbon is burned, cool the crucibles and cool the furnace.

(e) Dampen the ash with 50 percent nitric acid. Heat on a hot plate to eliminate acid. Replace the crucibles in the cooled furnace (below 80° C) and heat slowly to 550° C. Hold at 550° C about 30 minutes or until it is certain that all nitric acid fumes have been driven off. (If the furnace temperature is not uniform those crucibles near the door may not be as hot as some others. In this case, they should be removed and positions reversed. When this is necessary, two periods of about 30 minutes each are necessary.)

- (f) Remove crucibles from the furnace and allow to cool.

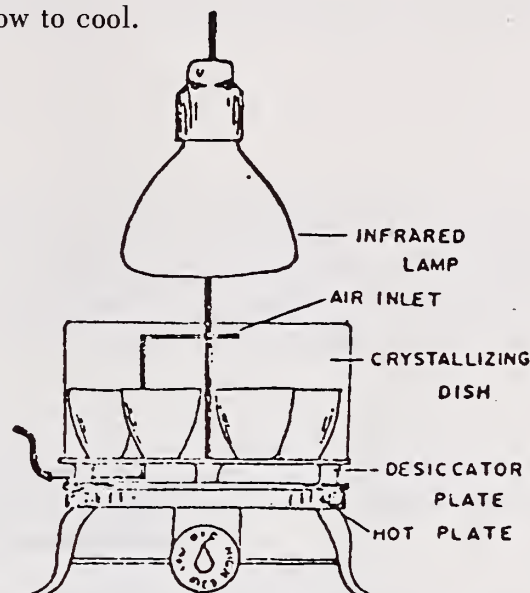


Figure 2.—Oven for pre-ashing hair samples.

5.009E. Sample Transfer

(a) Transfer the ash from the crucible to clean 125 ml Erlenmeyer flasks using flasks using four-10 ml portions of 4.5N hydrochloric acid.

(b) To this solution add 2 ml of 15% potassium iodide solution and mix well; let stand 15 min before analysis.

5.009F. Preparation of Standard Curve

(a) Into each of twenty 125 ml Erlenmeyer flasks pipette 40 ml of 4.5N hydrochloric acid.

(b) Into the flasks measure in duplicate the following: 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 10.0 μg of arsenic from the standards prepared above.

(c) Add to these solutions 2 ml of potassium iodide and mix well; let stand 15 min before analysis.

5.009G. Atomic Absorption Analysis

(a) Establish an argon-hydrogen-entrained air flame at the best conditions for arsenic analysis. (Flow rate about 17 liters/min for argon and 5 liters/min for hydrogen with the burner just below the optical path. It has been noted that lower noise levels and less sensitivity to drafts is experienced if the front of the flame chamber beneath the door is covered so that all air is drawn from beneath the instrument.)

(b) Add 2 pellets of NaBH_4 by placing them in the adapter (see Figure 3.) and letting them drop into the sample as connection is made.

(d) Measure the peak height and plot standard curve.

5.009H. Calculations

Standard curves are either calculated by least mean squares, for linear range only, or are plotted from the standard data obtained. The arsenic content of each sample is then determined from the standard curves.

NOTE: The calibration curve should be linear up to about 4.0 μg arsenic with some curvature at high arsenic levels. The calibration curves will vary considerably from one location to another depending on equipment, flow rates, etc.

References

1. E.F. Dalton and A.J. Malanoski, Atomic Absorption Newsletter Vol. 10, No. 4 (1971).
2. H.L. Kahn and J.E. Schallis, Atomic Absorption Newsletter 7, 5 (1968).
3. "Analytical Methods for Atomic Absorption Spectrophotometry," The Perkin-Elmer Corp., March, 1971.

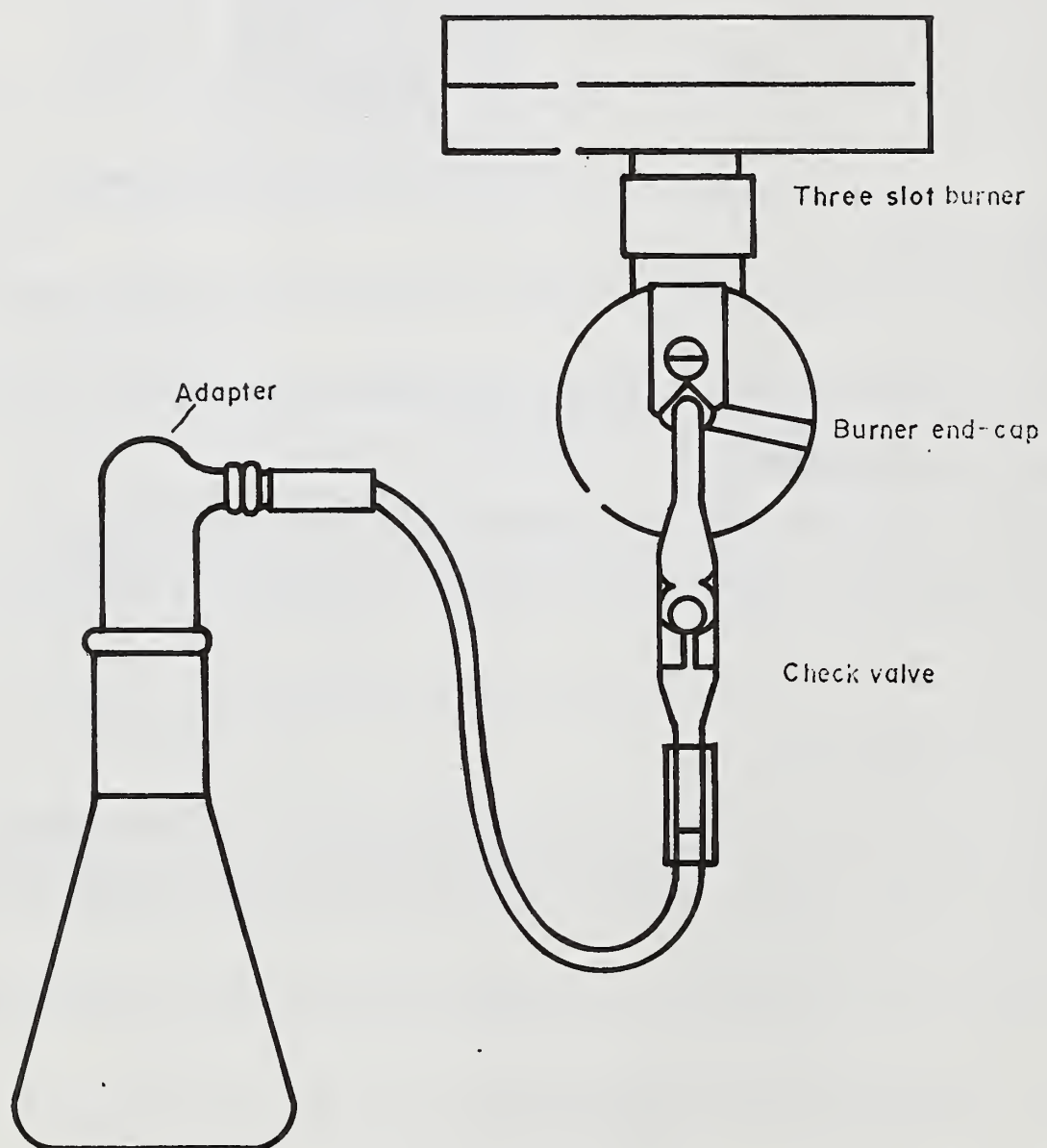


Figure 3.—Modification of instrument for arsenic analysis.

5.010 DETERMINATION OF TRACE ELEMENTS IN ANIMAL TISSUE BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

5.010A. Theory

The sample matrix is completely destroyed by treatment at elevated temperature in a suitable furnace. Generally, a second heat treatment is required to completely remove all traces of carbon residue. The ash residue is dissolved in HCl prior to analysis by atomic absorption spectrophotometry. This method is suitable for the analysis of copper (Cu), lead (Pb), zinc (Zn), cadmium (Cd), iron (Fe), nickel (Ni), cobalt (Co), and manganese (Mn), in liver, kidney or muscle tissue.

5.010B. Apparatus

- (a) Atomic absorption spectrophotometric instrument equipped with background correction capability and acetylene/air burner assembly. Perkin Elmer Model 603, or equivalent.
- (b) Drying oven — forced air drying oven capable of maintaining a temperature of $95^{\circ} \pm 5^{\circ}\text{C}$.
- (c) Ashing dishes — Vycor brand (50 ml), or equivalent (inner surfaces should not be etched).
- (d) Muffle furnace — capable of maintaining a temperature of $550^{\circ}\text{C} \pm 10^{\circ}\text{C}$.
- (e) Hot Plate — capable of maintaining surface temperature of $120^{\circ}\text{C} \pm 10^{\circ}\text{C}$.
- (f) Polyethylene centrifuge tubes.
- (g) Polyethylene bottles — 100, 250 ml — used for storing standard solutions.

5.010C. Reagents

- (a) Nitric acid — distilled from glass. G. Frederick Smith, or equivalent.
- (b) Hydrochloric acid, reagent grade.
- (c) 1N HCl — Dilute 83 ml conc HCl to 1L using good quality deionized water.
- (d) Dissolve 66.7 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 1L using good quality deionized water. (Soln. 1).
- (e) Dissolve 33.3 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 1L 1N HCl. (Soln.2).
- (f) Standard Solutions: Instrument calibration solutions

All standard solutions are made from commercially available reference standard solutions, use Fisher Certified Atomic Absorption Standards (or equivalent) for all metal standards used in this procedure. Concentrations of commercial standard solutions is typically 100 $\mu\text{g}/\text{ml}$. Make dilutions as appropriate, using Soln. #2 to reach working concentrations listed.

Metal	Std #1	Std #2	Std #3
	ug/ml	ug/ml	ug/ml
Zn	1.0	3.0	6.0
Mn	1.0	3.0	6.0
Fe	5.0	15.0	30.0
Cu	5.0	15.0	30.0
Ni	—	—	1.0
Co	—	—	1.0

Standard solutions for Pb and Cd are made separately. Starting with commercial standards of 1000 ug/ml, make appropriate dilutions using Soln #2. Separate standards for Pb and Cd are necessary due to trace contamination from other metal standards.

Metal	Std #1	Std #2	Std #3
	ug/ml	ug/ml	ug/ml
Pb	—	—	2.0
Cd	1.0	3.0	9.0

(g) Sample fortification solutions

Fortification standards are made from commercially available 1000 ug/ml certified standards. Use good quality deionized water to make appropriate dilutions. Add 1 ml redistilled nitric acid per liter.

Fortification standard #1 — Use 2.0 ml to fortify 15 g sample liver or kidney

Metal	ug/ml	Total ug in 2.0 ml	ppm in 15 g sample
Zn	240	480	32
Fe	240	480	32
Cu	240	480	32

Fortification Standard #2. Use 1.0 ml to fortify 15 g sample liver, kidney or muscle.

Metal	ug/ml	Total ug Added	ppm in 15 g sample
Co	10	10	0.67
Ni	10	10	0.67
Mn	20	20	1.33
Cu	20	20	1.33

Fortification Standard #3. Use 2.0 ml to fortify 15 g sample muscle.

Metal	ug/ml	Total ug Added	ppm in 15 g sample
Zn	240	480	32
Fe	240	480	32

Fortification Standard #4. Use 1.0 ml to fortify 15 g sample of liver, kidney or muscle.

<u>Metal</u>	<u>ug/ml</u>	<u>Total ug Added</u>	<u>ppm in 15 g sample</u>
Pb	50	50	3.33
Cd	50	50	3.33

5.010D. Sample Preparation

(a) Muscle tissue — Trim off as much fat as possible. Pass rapidly 3 times through a food chopper with plate openings no greater than $\frac{1}{8}$ ", mixing thoroughly after each chopping.

(b) Liver — Trim off as much connective tissue as possible. Place in a blender jar and blend until homogenous. To avoid overheating the liver, do not blend continuously for periods longer than 1 minute.

(c) Freeze samples if they are not to be analyzed immediately. (It is convenient to use a flat plastic bag to freeze sample in a slat 1-2 cm thick).

5.010E. Determination

(a) Weight $15.0 \text{ g} \pm 0.1 \text{ g}$ sample into 50 ml Vycor crucible. Add 7.5 ml Soln #1 and mix thoroughly.

(b) Place in drying oven set at $90^\circ - 95^\circ\text{C}$ until dry (about 6 hrs). The sample must be thoroughly dry at this point to prevent spattering.

(c) Place sample in cool ($<80^\circ\text{C}$) Muffle furnace. Raise temperature slowly ($2^\circ - 4^\circ\text{C}/\text{min}$) to 350°C . Hold at this temperature until smoking stops. Raise temperature slowly to $500^\circ - 550^\circ\text{C}$. The sample must not ignite. Hold at $500^\circ - 550^\circ$ for 16 hrs. Remove samples from oven and allow to cool.

(d) Generally, a second ashing step will be required to remove remaining carbon residue. Add 2 ml 50% HNO_3 taking care to wash down sides of crucible. Place sample on a hot plate set at 120°C to remove excess acid. Replace sample into cool Muffle furnace and again raise temperature to $500^\circ\text{C} - 550^\circ\text{C}$. Hold at that temperature for 1 hour. Repeat HNO_3 treatment if required to obtain carbon free ash.

(e) Add 15 ml 1N HCl and dissolve ash completely. Transfer to polyethylene tube for analysis.

(f) Set up AAS according to manufacturer's instructions. Insert a copper lamp and maximize the signal intensity by adjusting the position of the lamp. Make a fine tuning of the wavelength by maximizing the signal. Adjust the height of the burner. Turn on the flame and aspirate a copper solution ($5 \mu\text{g}/\text{ml}$ is convenient). Adjust the aspiration rate and position of the burner head for maximum signal. It should not be necessary to make changes for the other metals.

(g) Measure the absorbance for each metal in each standard and sample solution. It will usually be necessary to dilute (with Solution #2) the sample solutions in order to obtain absorbances for iron, zinc, and sometimes copper that are within the range of the standards.

5.010F. Calculations

Element concentrations are calculated directly by the AA spectrophotometer. With other types of instrumentation it may be necessary to construct a standard curve for each metal.

5.011 Determination of Clopidol in Liver and Muscle Tissues

Clopidol (3,5 dichloro-2,6 dimethyl-4-pyridinol) is effective as a coccidiostat and is formulated as a 25 percent premix called Coyden ® 25 (Dow Chemical). The premix is mixed with poultry feed to give a concentration of 0.0125 percent Clopidol.

5.011A. Principle

Poultry tissues are extracted with methanol, filtered and an aliquot taken through cleanup procedure employing an alumina and an anion exchange column. An aliquot is methylated in a closed system with diazomethane in a 70° C water bath. The excess reagent is evaporated, sodium hydroxide and water are added, and the mixture is extracted with benzene. An aliquot of the benzene layer containing the clopidol methyl ether (3,5-dichloro-4-methoxy-2,6-lutidine) is injected into a gas chromatograph equipped with an electron capture director. The peak height from the compound is compared to a standard curve prepared from pure methyl derivative.

SAFETY PRECAUTION

Diazomethane is toxic, can cause specific sensitivity, and is potentially explosive. Preparations, methylations and evaporations should be done in a hood. Avoid glass joints, etched or scratched glassware, and sharp edges. Diazomethane solutions should be stored in a freezer and should not be exposed to direct sunlight or strong artificial light. Prepare only the amount of diazomethane necessary, take precautions and use safety shields, and face shields.

5.011B. Apparatus

- (a) Grinder
- (b) Balance, top loader, (Mettler P1200 or equivalent)
- (c) Virtis homogenizer or equivalent and accessories
- (d) Filtering funnel—60ml, sintered glass disc, coarse (Kimax 28400 or equivalent)
- (e) Chromatographic column, 10mm id × 12cm, having a coarse fritted disc (Ace #5885-06 or equivalent)
- (f) Chromatographic column, 19mm id × 16cm, having a coarse fritted disc (Ace #5885-12 or equivalent)
- (g) Beakers and volumetric flasks as required
- (h) 70°C water bath
- (i) Screw cap culture tube with teflon faced cap liner, (Kimax 45066-A or equivalent)
- (j) Micro liter syringe
- (k) Diazomethane generator. Fire polished glass tubing, 2 rubber stoppers, 1-15ml test tube, 2 plain angle centrifuge tubes, 15ml capacity (Kimax 45159 or equivalent). (See construction under reagents)
- (l) Gas chromatograph with E.C. detector and 6 ft. 3mm id glass column packed with reagent 5.011C (m).

5.011C. Reagents

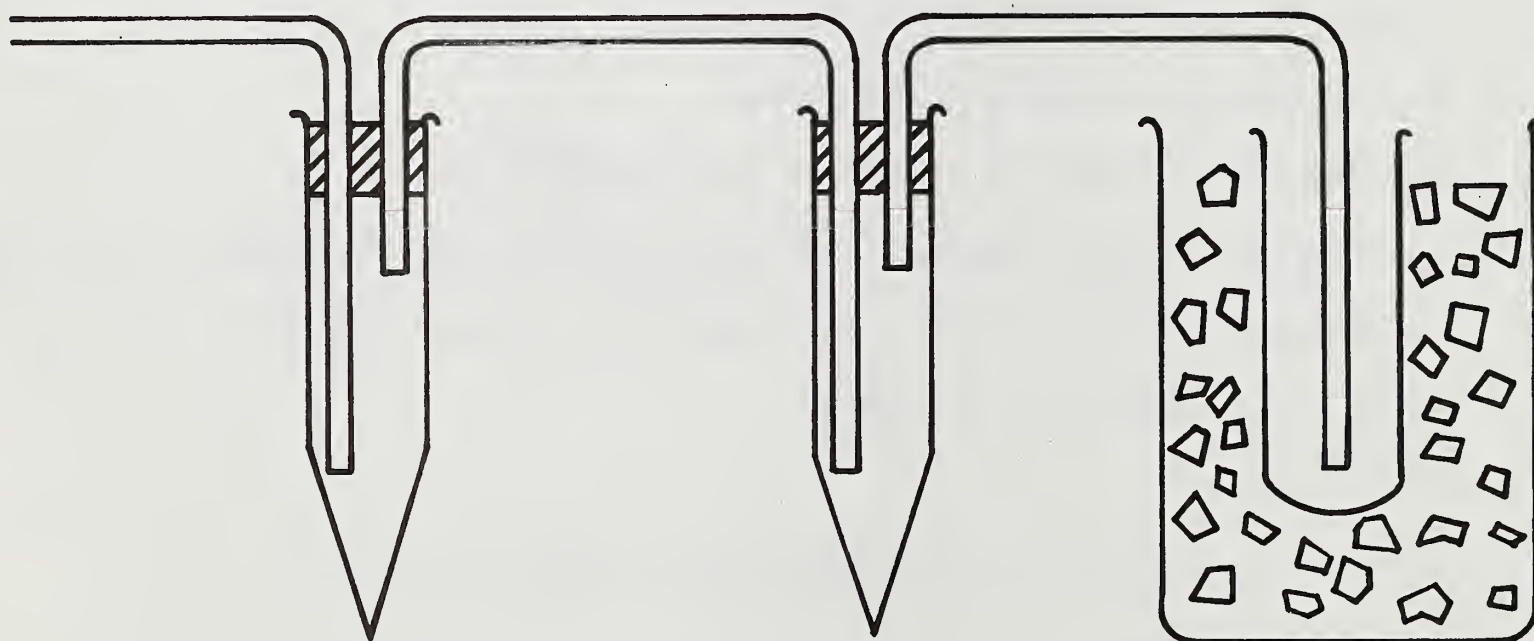
- (a) Alumina—Alcoa F-20, 80/100 mesh, Aluminum Company of America, East St. Louis, Ill., or equivalent.
- (b) Anion Exchange Resin—AGI-X8, 100/200 mesh, acetate form Bio-Rad Laboratories, Richmond, Calif., or equivalent.

- (c) Filter Aid, Hyflo Super-Cel or equivalent—Johns Manville Products Corp., New York, N.Y., or equivalent.
- (d) Methanol
- (e) 5 percent HCl in methanol
- (f) 80 percent methanol in water (prepare fresh daily)
- (g) Carborundum chips
- (h) 1 M Sodium Hydroxide - 4g per 100ml
- (i) Benzene

(j) Diazomethane: Prepare as described in Pesticide Analytical Manual, Vol. II, FDA, Washington DC, Sec. 120.227. Use the following proportions of reagents for reaction mixture: 300 mg Diazald®, n-Methyl-n-Nitroso-p-Toluene-Sulfonamide (available from Dow Chemical Co., Box 1706, Midland, MI 48640), 2.0ml ethyl ether, 2.0ml 60 percent KOH, 2.0ml Dowanol®, carbitol 2-(2-ethoxy-ethoxy-Ethanol (available from Dow Chemical Co., Box 1706, Midland, MI 48640).

Generate diazomethane by bubbling nitrogen through reaction mixture and collect for 5 minutes in 10ml ethyl ether trap submerged in ice bath. Store ethyl ether solution of diazomethane in the freezer.

DIAZOMETHANE GENERATOR (Fire polish all glass tubing)



(k) Clopidol Standard (Sampling coordinator, Ag.-Organics Dept., Dow Chemical USA, Box 1706, Midland, MI 48640).

(l) Clopidol Standard Solutions (for recovery samples)

Solution A, 100 µg/ml: Weigh 10.0 mg clopidol, transfer to 100 ml vol flask. Dilute to volume with methanol.

Solution B, 10 µg/ml: Dilute 10 ml Solution A to 100 ml with methanol.

Solution C, 1 µg/ml: Dilute 10 ml Solution B to 100 ml with methanol.

(m) Column packing: 25 percent DC-200 silicone oil or equivalent on Chromosorb W-AW 80/100 mesh (Applied Science Laboratories, State College, PA).

(n) 3,5-Dichloro-4-methoxy 2,6-Lutidine, analytical standard (Sampling Coordinator, Ag-Organics Dept., Dow Chemical USA, Box 1706, Midland, MI 48604).

(o) 3,5-Dichloro-4-methoxy 2,6-Lutidine standard solutions (for standard curve):

Solution D: Clopidol equivalent 1000 $\mu\text{g/ml}$ -Weigh 0.1073 g, dissolve, transfer and dilute to volume in 100 ml vol flask with benzene.

Solution E: Clopidol equivalent 10 $\mu\text{g/ml}$. Dilute 1 ml solution D to 100 ml with benzene.

Solution F: Clopidol equivalent 0.2 $\mu\text{g/ml}$. Dilute 2 ml solution E to 100 ml with benzene.

Solution G: Clopidol equivalent 0.15 $\mu\text{g/ml}$. Dilute 1.5 ml solution E to 100 ml with benzene.

Solution H: Clopidol equivalent 0.10 $\mu\text{g/ml}$. Dilute 25 ml solution F to 50 ml with benzene.

Solution I: Clopidol equivalent 0.05 $\mu\text{g/ml}$. Dilute 25 ml solution H to 50 ml with benzene.

Solution J: Clopidol equivalent 0.02 $\mu\text{g/ml}$. Dilute 10 ml solution H to 50 ml with benzene.

Solution K: Clopidol equivalent 0.01 $\mu\text{g/ml}$. Dilute 5 ml solution H to 50 ml with benzene.

5.011D. Standard Curve: (Prepare Daily)

Condition GC column by injecting replicate aliquots of solution F until the peak height is the same for consecutive injections. To prepare the standard curve inject 3 μ l each of solutions F through K and plot peak height (mm) or area versus μ g clodolol equivalent/ml.

5.011E. Gas Chromatography

(a) Column preparation—Condition column for 18 hours or more at 200° C with 75 to 100 ml/min nitrogen carrier gas.

(b) Typical operating conditions (Barber Coleman 5000)

(1) Carrier gas—prepurified nitrogen 120 ml/min.

(2) Column temperature—180° C.

(3) Detector temperature—220° C.

(4) Injector temperature—220°C.

(5) Detector operating voltage—38 volts.

(6) Electrometer—adjust for optimum response

Retention time—approximately 4 min.

5.011F. Determination

(a) Weigh 20 g ground tissue (liver is the tissue of choice) into Vertis jars. Run two blank tissues with each analytical set. One is for the recovery; the other is used as a blank (G').

(b) Add 2 ml solution C to one blank tissue for 0.1 ppm recovery or 1 ml solution B for 0.5 recovery.

(c) Add 12 g Hyflo Super-Cel to liver tissues and 2 g Hyflo Super-Cel to muscle tissues.

(d) Add 50 ml methanol and blend for 3 min.

(e) Filter through 50 ml sintered glass funnel prepared with 2 g Hyflo Super-Cel pad using vacuum flask. Wash with methanol until about 100 ml is collected. Dilute to 100 ml.

(f) Prepare alumina column with 6 g Alcoa F-20 alumina into 19 mm id \times 16 cm column. Rinse with 10 ml methanol.

(g) Prepare anion exchange column, using 10 mm id \times 12 cm column. Slurry the AG1-8 resin with methanol. Add 1 cm resin (after settling). Rinse with 2 ml methanol.

(h) Place the alumina column over the exchange resin column with collection beaker underneath allowing flow through both columns.

(i) Pipet 20 ml of sample extract onto alumina column. Wash with 10 ml methanol.

(j) Remove the alumina column and replace collection beaker with 25 ml volumetric flask.

(k) Elute with 2 \times 10 ml portions of 5 percent HCl in methanol. Dilute to 25 ml with methanol.

- (l) In 70°C water bath, evaporate 1 ml aliquot just to dryness in screw cap tube using small jet of air.
- (m) Add 0.2 ml 80 percent methanol (fresh daily). Warm to dissolve residue.
- (n) Add 1 ml diazomethane, cap, and heat for 2 min. in 70° C water bath. Remove and let sit 5 min.
- (o) Add small piece of carborundum and evaporate ether layer *very carefully* just touching tip of tube to surface of 70°C water bath.
- (p) Add 0.1 ml 1 M NaOH, 5 ml water and 1 ml benzene.
- (q) Cap and shake vigorously for 1 min. Let settle and inject 3 µl benzene layer being careful to avoid aqueous layer.
- (r) Determine the peak height and interpolate from the standard curve the µl/ml of clopidol represented in the benzene extract.

5.011G. Calculations

$$R \text{ (recovery)} = \frac{\text{ppm clopidol found}}{\text{ppm clopidol added}}$$

$$\text{ppm clopidol} = \frac{6.25 (G - G')}{R}$$

$$6.25 = \text{dilution factor i.e. } \frac{100 \text{ ml} \times 25 \text{ ml} \times 1 \text{ ml}}{20 \text{ g} \times 20 \text{ ml} \times 1 \text{ ml}}$$

G = µg/ml found in sample using the standard curve.

G' = µg/ml found in blank using the standard curve.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

5.012 Quantitative Assay for the Primary Hydroxy Metabolite of Iprnidazole (IprOH)

5.012A. Theory:

Sample preparation is based on a conventional extraction and cleanup scheme. Quantitation is accomplished by capillary gas chromatography with electron capture detection. This procedure was validated for turkey and swine muscle fortified at 0, 1, 2, and 4 ppb. Quantitative results are corrected for tissue "background". Average recovery exceeds 60 percent.

5.012B Apparatus:

- (a) Perkin Elmer Sigma 2000 Gas Chromatograph (or equivalent) equipped with a split/splitless capillary injector and a ^{63}Ni electron capture detector.
- (b) Gas chromatographic column: 50 meter, 5 percent phenylmethyl silicone coated, film thickness 0.33 μm , 0.25 mm I.D., HP Ultra 19091, (b) option 105, or equivalent.
- (c) Tekmar Model SDT Tissuemizer or equivalent.
- (d) IEC PR-6000 Centrifuge or equivalent.
- (e) pH meter, Corning 125 or equivalent.
- (f) Rotary Evaporator, Buchi Rotovapor R110 or equivalent
- (g) N-Evap, Organomation Model 111 or equivalent
- (h) Glass columns, 1 cm \times 25 cm with 200 ml reservoir
- (i) Vortex mixer
- (j) Separatory funnels, 125 ml
- (k) Centrifuge bottles, 250 ml
- (l) Centrifuge tubes, 50 ml, 15 ml, and 2 ml
- (m) GC Protector, J&W Scientific Inc.
- (n) Vacuum Manifold

5.012C. Reagents

- (a) Toluene, distilled in glass, Burdick & Jackson, Inc.
- (b) Ethyl Ether, distilled in glass, Burdick & Jackson, Inc.
- (c) Hexane, distilled in glass, Burdick & Jackson, Inc.
- (d) Chloroform, distilled in glass, Burdick & Jackson, Inc.
- (e) Methylene Chloride, distilled in glass, Burdick & Jackson, Inc.
- (f) Methanol, distilled in glass, Burdick & Jackson, Inc.
- (g) Sodium Hydroxide, pellets, reagent, ACS

- (h) Sodium Chloride, reagent, ACS
- (i) Hydrochloric Acid, reagent, ACS
- (j) Sodium Tetraborate, decahydrate, reagent, ACS
- (k) Silica gel, 60-200 mesh, J.T. Baker or equivalent.
- (l) C-18 disposable extraction columns, 3 ml volume coupled to 75 ml column reservoir, J.T. Baker or equivalent.
- (m) Diatomaceous Earth (Kieselguhr), EM Science, #11738-1

5.012D Preparation of Chromatographic Columns

(a) Silica gel pretreatment

- (1) Wash silica gel with MeOH by gently swirling 20-30 g of 60-200 mesh silica gel in 200 ml of MeOH in a 500 ml Erlenmeyer flask for 2-3 minutes.
- (2) Filter using vacuum suction until silica gel appears dry and has no solvent odor.
- (3) Activate overnight @ 110° in mechanical convection oven.
- (4) Store in desiccator and heat for 1 hour at 110° prior to use.

(b) Silica gel column

- (1) Slurry 2 g of silica gel with toluene in a small beaker and transfer to a 1 cm × 20 cm glass column containing a small plug of silanized glass wool at the bottom.
- (2) Allow silica to settle and add 0.5 g diatomaceous earth.
- (3) Allow toluene to pass through the column.

5.012E Standard Solutions

NOTE: All standards should be protected from direct UV light. Use of actinic glassware and refrigeration is recommended for storage of standards. Under these conditions the stock and working standards remained stable for extended periods.

(a) Stock Standard: 1 mg IprOH/ml MeOH

Accurately weigh 10 mg of reference standard and place it in a small 10 ml beaker. Dissolve the IprOH in 1 to 2 ml of MeOH and transfer to a 10 ml actinic volumetric flask. Rinse beaker with 2 to 3 additional ml of MeOH. Transfer rinse to the volumetric and bring to volume with MeOH.

(b) Working Standard: 10 µg/ml toluene

Into a 10 ml low actinic volumetric flask pipette 100 µl of stock standard (1 mg/ml) and bring to volume with toluene.

5.012F. Sample Extraction and Cleanup

(a) Extraction

- (1) Weight 50 ± 0.5 g of frozen, preground, muscle tissue into a 250 ml heavy wall glass centrifuge bottle.

NOTE: Each sample set must include a control and fortified control sample.

- (2) Add 4 g of sodium chloride and 4 g of sodium tetraborate.
- (3) Add 100 ml of toluene and blend for 30 to 40 seconds until homogeneous.
- (4) Centrifuge at 1750 - 1800 rpm for 10 minutes.
- (5) Filter (using Reeve Angel 802) into a 1000 ml round bottom flask
- (6) Repeat step (a) 3 through (a) 5, combining filtrates.
- (7) Rotary evaporate the toluene extract to an oily residue of approximately 5 ml.

NOTE: Losses during evaporation can be substantial. The analyst must verify that the conditions used are not too harsh. Conditions will vary based on: the type of condenser used, the amount of vacuum applied, the temperature of the water bath, and the surface area and speed (rpm) of the round bottom flask in the water bath. DO NOT take to dryness.

- (8) Reconstitute the oily residue in 5-10 ml of toluene.
- (9) Transfer to column with a 10 ml glass syringe then repeat twice more, using 5-10 ml rinses, apply each rinse to head of column.

(b) Cleanup

- (1) Allow the column to drain, slight N_2 pressure may be used if necessary.
- (2) Wash the column with 10 ml of hexane followed by 10 ml of chloroform.
- (3) Elute the silica gel column with 30 ml of water-saturated ethyl ether.
- (4) Add 5 ml of 1N HCL to the water-saturated ethyl ether and shake for 30 seconds, then centrifuge 5 minutes @ 750 rpm.
- (5) Aspirate and discard the ethyl ether.
- (6) Wash the aqueous acid with one 20 ml portion of hexane. Aspirate and discard the hexane layer.
- (7) Adjust the pH of the aqueous phase to pH 6.0 to 7.5 and dilute to 10 ml with Millipore purified water.
- (8) Load aqueous phase on C-18 extraction column. NOTE: Columns must be prepared by passing 3 ml MeOH followed by 3 ml water through them before use.
- (9) Elute C-18 column with 12 ml of MeOH: water 35:65.
- (10) Adjust pH of aqueous eluent to pH 9-11 and immediately extract with 2×10 ml methylene chloride.
- (11) Combine the methylene chloride extracts in a 50 ml centrifuge tube.
- (12) Concentrate methylene chloride to approximately 2 ml and transfer to a 2 ml graduated concentration tube.
- (13) Continue concentration of $MeCl_2$ until final volume is 100 to 200 μ l. Do not concentrate to dryness.
- (14) Add 300 to 400 μ l of toluene and continue to concentrate until volume is less than 300 μ l.

(15) Adjust final volume to 300 ul with toluene. Sample is now ready for GC analysis.

5.012G Gas Chromatographic Instrument Parameters

(a) Gases

(1) Carrier: Hydrogen (GC oven protector required), head pressure 25-30 psi, linear velocity 330 cm/sec.

(2) Makeup gas to ^{63}Ni ECD—Ar:CH₃ 95:5, flow measured at detector: 60 ml/min.

(3) Septum vent: 2 ml/min.

(4) Purge vent: 40 ml/min.

(b) Temperatures

(1) Injector: 200°C

(2) Oven: 80°C for 2 minutes program to 180° @ 30°/min., hold for 10 minutes, then increase to 200° and hold for 5 minutes.

(3) Detector Temp 300–350°C

(c) Splitless Injection

(1) Purge valve closed at injection

(2) Purge valve opened 60 sec. after injection

5.012H Quantitative Assay of Tissue Workup

(a) Prepare a series of dilutions of the IprOH working std. section 5.012E 2 (10 ng/ul) as follows:

(1) working std 1/10, 1.00 ng/ul

(2) working std 1/20, 0.50 ng/ul

(3) working std 1/40, 0.25 ng/ul

(4) working std 1/100, 0.10 ng/ul

Based on a 50 g sample and a 300 ul final volume, the tissue equivalences for the standards are:

(i) 6.0 ppb

(ii) 3.0 ppb

(iii) 1.5 ppb

(iv) 0.6 ppb

(b) Inject an equal volume (2-3 ul) of each standard and record the response. IprOH should elute between 11-13 minutes based on the conditions outlined in section 5.012G.

(c) Generate a least squares linear regression standard curve; $Y = Mx + b$, by plotting the GC response vs. the concentration of each standard.

$Y = \text{Response}$

$M = \text{Slope}$

$X = \text{Conc.}$

$b = \text{Intercept}$

(d) Inject equal volumes (2-3 ul) of:

(1) Control sample workup

(2) Fortified recovery workup

(3) All tissue workups

(e) Reinject at least one of the standards to verify that the stability of the chromatographic system was maintained during data generation.

NOTE: All injections must be of the same volume.

5.012I. Calculation

(a) Quantitative values are calculated by substituting the appropriate response value (Y) into the least squares linear regression curve described in section 5.012H, $Y = MX + b$ where: Y = Response at proper retention time; X = Unknown conc. to be determined; M = Slope as determined by standard curve; b = Intercept as determined by standard curve.

(b) Calculate the "method/tissue background" concentration based on the response of the control sample:

$$Y \text{ Background} = mX \text{ Background} + b$$

(c) Calculate the recovery concentration based on the fortified recovery response.

$$Y \text{ Total} = mX \text{ total} + b$$

$$X \text{ Recovery} = X \text{ Total} - X \text{ Background}$$

(d) Calculate the percent recovery as follows:

$$\% \text{ Recovery} = \frac{X \text{ Recovery}}{X \text{ std @ same conc.}} \times 100$$

(Recovery should exceed 60%)

(e) Calculate the sample workup concentration as follows:

$$Y \text{ Sample} = mX \text{ Sample} + b$$

(f) Calculate tissue equivalency

$$\begin{array}{l} \text{Tissue} \\ \text{Conc.} \end{array} = X \text{ Sample} - X \text{ Background}$$

5.012J Stopping Points:

The ideal situation is to start a set of samples at the beginning of the day, then to use an automatic sampler to inject the samples on the GC overnight. If the analysis cannot be completed in one day, acceptable stopping points are 5.012F 2e, f, l and o. Care should be taken such that the sample workup *NOT* be allowed: to be taken to dryness, to be exposed to UV light for extended periods, to stand in solution above pH 9 and to stand in methylene chloride solvent for extended periods.

5.013 Hydroxyipronidazole Gas Chromatography/Mass Spectrometry Confirmatory Procedure

5.013A. Theory

This is a method to confirm the presence of hydroxyipronidazole (IPrOH) in swine, beef, and poultry muscle tissue by quadrupole GC/MS. The method uses a capillary column for separation and negative ion chemical ionization (NICI) for detection/confirmation. The method is sensitive to 100 pg of IPrOH injected onto the column. The aliquot remaining from the determinative step is generally suitable for confirmatory use. (The action level for IPrOH is 2 ppb).

5.013B. Apparatus

- (a) Instrument: Finnigan 4000 GC/MS with capillary column capability, or equivalent.

GC Conditions and Parameters

Column: 25 meter OV-1 fused silica capillary column.

Injector: 220°C

GC oven temperature: 150°C (held 1 minute) then programmed to 250°C at 20°C/minute, and then held 1 minute.

Vent delay: 1 minute, solvent (toluene) elutes in 2 minutes.

Column pressure: 16 psi.

Mass Analyzer Parameters

With a Finnigan 4000 GC/MS system the following instrumental parameters are used:

Ionizing reagent gas: methane (CH₄).

Pressure: 4.5×10^{-5} Torr.

Manifold temperature: 85°C.

Ionizer temperature: 250°C.

Electron energy: 70 eV

Emmision current: 550 mA

Dynode voltage: 2.6 kV

Electron multiplier voltage: $2000 \text{ eV} \times 10^{-8} \text{ A/V}$ for 100-600 pg of IPrOH

- (b) 2 Syringes, 10ul
- (c) Syringe cleaning device - See 5.013G. (a). below

5.013C. Reagents

- (a) See Reagents Section of method 5.012.
- (b) Methanol — Burdick and Jackson, distilled in glass, or equivalent.

(c) Acetone — Burdick and Jackson, distilled in glass, or equivalent.

(d) Hexane — Burdick and Jackson, distilled in glass, or equivalent.

(e) Toluene — Burdick and Jackson, distilled in glass, or equivalent.

5.013D. Calibration of Mass Analyzer

With system in *EI* mode and calibration gas off, m/z 28 and 32 are centered. Calibration gas is turned on and m/z 219 is monitored. After centering m/z 219, the system is placed in the NCI mode. NCI mode calibration is accomplished by monitoring, maximizing, and centering m/z 333 and 414. The amount of CH_4 being introduced is adjusted such that a low mass ion (between m/z 100 and 200) is maximized. The ratio of 333:414 should be approximately 15:100. If low mass sensitivity decreases by more than 50 percent and re-tuning does not improve the situation, the source should be cleaned.

5.013E. Acquisition of Mass Spectral Data

After calibration, 1 ng of IPrOH standard is injected. A mass spectrum of the standard is produced by scanning from 50 to 200 amu. The six most intense ions characteristic of IPrOH are selected for specific ion monitoring. These ions are m/z 186, 185 (M^-), 170, 153, 135, and 127. The monitored ions are believed to be characteristic of the following fragmentation pathways.

<u>m/z</u>		<u>Fragmentation Product</u>
185 ⁻	=	M^-
186 ⁻	=	^{13}C isotope of M^-
170 ⁻	=	$[\text{M}^- - \text{CH}_3]$
153 ⁻	=	$[\text{M}^- - \text{CH}_3\text{OH}]$
135 ⁻	=	$[\text{M}^- - \text{CH}_3 - \text{CH}_3\text{OH}]$
127 ⁻	=	$[\text{M}^- - \text{CH}_3 - \text{CH}_3\text{OH} - \text{CN}]$

5.013F. Confirmatory Criteria

The retention time of IPrOH is approximately 5 minutes under the described parameters. Retention time of the IPrOH component peak for three (3) standard injections must be reproducible within ± 0.25 minutes. The ratios of m/z 186/185, 170/185, 153/185, 135/185, and 127/185 from three standard injections must be determined and reproducible within ± 10 percent. This data must be obtained at the tolerance level, and at the IPrOH level thought to be present in the suspect sample.

Confirmation of real samples requires the presence of m/z 185, 186 and at least three of the other four ions monitored. Three ion ratios (relative to m/z 185) must be within 10 - 15 percent of that found for the standard at similar levels of interest.

5.013G. Injection Procedure

(a) In both the determinative and confirmatory procedures IPrOH carryover originating from the syringe has been observed. Simple syringe rinsing is inadequate. To resolve this problem a simple vacuum apparatus is employed to clean the syringe.

The syringe cleaning apparatus consists of a side-arm (Buchner) flask connected to a vacuum system. The flask is stoppered with a one hole rubber stopper. Vacuum applied to the side-arm creates suction through the hole in the stopper.

A syringe (plunger removed) is placed over the hole (needle up). Suction is applied through the syringe needle and barrel. A pasteur pipette (with a small squeeze bulb) is filled with solvent (1-2 ml toluene). The pipette tip is placed over the upright syringe needle and solvent is drawn through the syringe needle and barrel. Leaving the pipette over the syringe needle after the solvent is drawn out of the pipette allows air to dry the syringe and provides a barrier which prevents dust particles from being drawn into the syringe during drying.

If cleaning is unable to remove the interference, contamination of solvents may have occurred. New solutions should be prepared. To minimize ghosting or carry-over problems, inject series of samples and standards in order of increasing concentration.

(b) Two ten microliter syringes are necessary for efficient analysis of a sample set. Syringes must be rinsed sequentially with methanol, acetone, and hexane. After use, the syringe is rinsed with the standard/sample solvent (toluene). Prior to injecting a standard or sample, a solvent/syringe check is accomplished by injection of 5ul of the solvent (toluene). This injection is monitored as any other standard/sample run. Data (i.e., ion fragment ratios) are valid only when a solvent/syringe check (immediately preceding a standard or sample injection) has demonstrated zero response for m/z 185 at the retention time expected for IPrOH. Inject standard to determine the retention of IPrOH.

(c) The sequence of analyses will follow this pattern:

- (1) 1 ng IPrOH standard. full scan acquisition (50-200 amu)
- (2) 5 ul solvent, MID acquisition (solvent/syringe check)
- (3) 1 ul. 4 ppb standard, 666 pg, MID acquisition
- (4) 5 ul solvent, MID acquisition (solvent/syringe check)
- (5) 1 ul, 2 ppb standard, 333 pg, MID acquisition
- (6) 5 ul solvent, MID acquisition (solvent/syringe check)
- (7) 1 ul, 1 ppb standard, 166 pg, MID acquisition
- (8) 5 ul solvent, MID acquisition (solvent/syringe check)
- (9) Obtain acceptable ratios for series of three analyses of IPrOH standard

Notes: It has been our experience that a contaminant may persist at low levels of detection. The contaminant will appear near the retention time of IPrOH. This component is believed to be a phthalate by virtue of an intense m/z 148 (by NCI). Experience also indicates a related compound, hydroxydimetridazole (DMOH), will be considered as a "presumptive positive" in the determinative procedure if present in sufficient quantity. This component would not be observed (zero response) under GC/MS conditions for selected ion monitoring of IPrOH. The molecular anion of DMOH is m/z 157 and should be monitored when an IPrOH "presumptive positive" sample cannot be confirmed by GC/MS. This procedure would only be undertaken if IPrOH can not be confirmed, as part of a procedure to identify DMOH.

5.014 Determination of Carbadox

This method is applicable to the determination of the carbadox metabolite (quinoxaline-2-carboxylic acid) in swine tissues.

Note: Because this analytical procedure is the official method, all positive samples requiring regulatory action must be analyzed by this procedure. For routine screening, the FDA approved New Animal Drug Application (NADA) procedure may be used. See References (2) and (3).

5.014A. Theory

A tissue sample is hydrolyzed in an alkaline medium, cooled and the hydrolyzate acidified. The hydrolyzate is extracted three times with ethyl acetate. The combined extracts are washed with a citric acid buffer, acidified with acid and partitioned with benzene. The benzene fraction is dried and the residue reacted with a n-propanol + sulfuric acid mixture to form the propyl ester.

The propyl ester is concentrated and applied to a thin-layer plate. The plate is developed and the sample zone identified. The sample zone is removed from the plate and the ester is eluted from the absorbent with ethyl acetate and quantified by gas-liquid chromatography using an electron-capture detector.

5.014B. Apparatus

- (a) Column, glass-tapered at one end, 0.9 centimeters \times 21.5 centimeters, prepared from a 10 milliliter serological pipette.
- (b) Centrifuge tubes, heavy duty 50 ml graduated (60 ml capacity), equipped with glass stoppers, R. C. Ewald, Inc., or equivalent.
- (c) Centrifuge tubes, 50 ml graduated, equipped with glass stoppered.
- (d) Volumetric flasks, 5-, 10-, 100- and 250-ml capacity, glass-stoppered.
- (e) Pipettes, automatic transfer, 10-, 15-, and 25-ml delivery volume.
- (f) Pipettes, measuring, 0.1 and 0.5 ml delivery volume.
- (g) Pipettes, volumetric 1-, 2-, 3-, 4, and 5-ml delivery volume.
- (h) Pipette, serological—10 ml delivery volume.
- (i) Pipettes—Pasteur, disposable.
- (j) Propipette bulb.
- (k) Syringe—10 μ l capacity, Hamilton or equivalent.
- (l) Crystallizing dish—190 mm (diameter) \times 100mm (height), for oil bath.
- (m) Test tube rack.
- (n) Test tube mixer—Vortex mixer or equivalent.
- (o) Lab jack—Cenco or equivalent.
- (p) Thermo-stir hotplate.

- (q) Magnetic stirrer bar (Teflon®).
- (r) Thermometer, 0° to 150°C range.
- (s) Knife (for cutting frozen tissue).
- (t) Ultraviolet light, 254 nanometers and 365 nanometers.
- (u) Scalpel.
- (v) Torsion balance-style RX-1, class A, Torsion Balance Co., or equivalent.
- (w) Cahn electrobalance: Cahn Model C-2 or equivalent.
- (x) Centrifuge: International, size 2, model K, or equivalent.
- (y) Rotary evaporator equipped either with a water aspirator or with a vacuum pump and condenser.
- (z) Alkacid test paper.
- (aa) Glassine paper.
- (bb) Glasswool.
- (cc) Flask—round bottom, 29/42 ST, 250 ml.
- (dd) Flask—round bottom, 19/22 ST, 65 ml.
- (ee) Funnel—burette.
- (ff) Hair dryer.
- (gg) pH meter.
- (hh) Tray—instrument, stainless steel.
- (ii) Water bath.
- (jj) Precoated thin layer plates—20 × 20 cm; 250 μm thickness, Silica gel GF, E. Merck, Darmstadt; distributed by Brinkmann Instruments Inc., Westbury, NY 11590 or equivalent.
- (kk) Desaga multiplate developing tanks for five 20 × 20 cm plates—distributed by Brinkmann Instruments Inc. or equivalent.
- (ll) Gas-liquid chromatograph—Tracor model 220 or equivalent, equipped with a ⁶³Ni electron affinity pulsed detector and a 0-1 MV recorder. Conditions and operating parameters for the gas-liquid chromatograph are: Isothermal column temperature 175°C; inlet heater, 270°C; EC detector temperature, 275°C; argon + methane (95:5) flowrate, 100 ml/min (40 psi); chart speed, ½-inch per minute, attenuation, 10 × 64. Detector pulse parameters: RF mode; voltage output, 55; pulse rate, 270 microseconds; pulse width, 3.0 microseconds. A glass sleeve injection port liner is installed for off-column injections.
- (mm) Packing—3 percent OV-17 on Gas Chrom Q, 60-80 mesh, Applied Sciences Laboratories, Inc. or equivalent.
- (nn) Column: Pyrex glass, U-tube, 6 feet × 4 millimeters (id). Condition the packed column at 280°C for at least 70 hours with argon-methane (95:5) flow, detached from the detector input.

(oo) Septum: High temperature type (HT-13), Applied Sciences Laboratories, Inc. or equivalent.

(pp) Detector-⁶³Ni electron capture. The voltage current profile for this detector should plateau at 30 volts or less in the DC mode when a stream of nitrogen gas is passed through the column and the electron capture detector.

5.014C. Reagents

(a) Benzene: Distilled-in-Glass grade, or equivalent.

(b) Ethyl acetate: Distilled-in-Glass grade, or equivalent.

(c) n-Hexane: Distilled-in-Glass grade, or equivalent.

(d) 1-Propanal: Reagent grade, dried over molecular sieve pellets (5A).

(e) Citric acid monohydrate: U.S.P., Pfizer, Inc., or equivalent.

(f) Potassium hydroxide: Pellets, reagent grade.

(g) Sodium hydroxide: Pellets, reagent grade.

(h) Hydrochloric acid: Reagent, A.C.S.

(i) Sulfuric acid: Reagent, A.C.S.

(j) Sodium sulfate: Anhydrous, reagent grade.

(k) Quinoxaline: 2-carboxylic acid—Pfizer Inc., or equivalent.

(l) Propyl quinoxaline: 2-carboxylate—Pfizer, Inc., or equivalent.

(m) Acridine: Practical grade; Matheson Coleman and Bell or equivalent.

(n) 1M Citric acid.

(o) 5M Sodium hydroxide.

(p) 3M Potassium hydroxide.

(q) 0.5M Citric acid buffer: Adjust the pH of 100 ml of 1 M citric acid to pH 6.0 with 5M sodium hydroxide (approximately 55 ml), using a previously calibrated pH meter. Adjust the final volume to 200 ml with distilled water. Before making the final pH adjustment, cool the buffer to room temperature.

(r) 1-Propanol-sulfuric acid reagent (97:3). Dilute 3 ml of concentrated sulfuric acid to 100 ml with dried, filtered, and cooled 1-propanol.

(s) Acridine solution. Dissolve 1 mg of acridine in 100 ml of benzene.

(t) Quinoxaline-2-carboxylic acid solutions:

(1) Stock solution A: Dissolve 1.25 mg of quinoxaline-2-carboxylic acid in enough 1-propanol to make 100.0 ml (concentration 12.5 µg/ml).

(2) Stock solution B: Dilute 1.0 ml of stock solution A to 100.0 ml with 1-propanol-sulfuric acid reagent (concentration 0.125 µg/ml).

(3) Working standard solution C: Dilute a 2.0 ml aliquot of stock solution B to 10.0 ml with 1-propanol-sulfuric acid reagent (concentration 25.0 ng/ml).

(4) Working standard solution D: Dilute a 3.0 ml aliquot of stock solution B to 10.0 ml with 1-propanol-sulfuric acid reagent (concentration 37.5 ng/ml).

(5) Working standard solution E: Dilute a 4.0 ml aliquot of stock solution B to 10.0 ml with 1-propanol-sulfuric acid reagent (concentration 50.0 ng/ml).

(6) Fortification solution: Dilute 3.0 ml stock solution A to 250 ml with distilled water (concentration 150 ng/ml).

(7) Propyl quinoxaline-2-carboxylate solution: Dissolve 1.00 mg of propyl quinoxaline-2-carboxylate in enough ethyl acetate to make 10 ml (concentration 100 µg/ml).

5.014D. Determination

(a) Dissolution and Hydrolysis Step

Transfer 5 grams of swine tissue (freshly sliced from frozen tissue) to a 50 ml centrifuge tube. Add 10 ml of 3M potassium hydroxide, stopper, and place in a 100°C silicone oil bath for 1 hour.

NOTE: The level of the silicone oil bath should exceed that of the tissue sample. Stopper the tubes lightly in order to allow the digestion mixture to "breathe." To determine the recovery of quinoxaline-2-carboxylic acid in swine tissue at the 30 ppb level, fortify 5 g of sample with 1 ml of fortification solution (concentration 150 ng/ml).

(b) Extraction Step

(1) Cool the alkaline hydrolyzate in an ice bath and acidify to \leq pH 1 (deep red to alkacid test paper) with 4 ml of concentrated hydrochloric acid. Add 15 ml of ethyl acetate to the acidified hydrolyzate, stopper, and extract by shaking for 20 seconds. Centrifuge the mixture at 1,500 revolutions per minute for 5 minutes to clarify the ethyl acetate phase. Recover the ethyl acetate phase with a blowout pipette equipped with a propipette bulb, and transfer this extract to a 60-ml separatory funnel equipped with teflon stopcocks. Re-extract the hydrolyzate with two additional 15-ml portions of ethyl acetate, and combine the organic extracts.

NOTE: Do not contaminate the ethyl acetate phase with interfacial material during these extractions. Quinoxaline-2-carboxylic acid is unstable in strongly acidic solutions. Continue to process these extracts through the benzene extraction and evaporation steps.

(2) Add 5 ml of 0.5M citric acid buffer (pH 6.0) to the ethyl acetate extract, shake, and allow the lower phase to clarify for about 20 minutes. Collect the aqueous phase in a 50 ml glass-stoppered centrifuge tube. Reextract the ethyl acetate phase with an additional 5 ml of pH 6 buffer, wait for the aqueous phase to clarify, and combine the aqueous extracts. Acidify (\leq pH 1) the aqueous extract with 2 ml of concentrated hydrochloric acid, stopper, and extract with 25 ml of benzene. Centrifuge to clarify the benzene layer and transfer the organic phase, using a blowout pipette equipped with a propipette bulb, to a 250 ml round bottom flask. Repeat the extraction and centrifugation steps three times. Combine the benzene extracts (about 100 ml) and evaporate to near-dryness, using a rotary evaporator equipped with a water aspirator and with a water bath set at 40°C.

NOTE: A rotary evaporator equipped with a vacuum pump and condenser may be used at this point. These residues may be stored overnight.

(c) Esterification Step

Reconstitute the residue from the previous step by rinsing the walls of the round bottom flask with 2×2 ml of 1-propanol-sulfuric acid reagent: transfer each rinse with a disposable pipette to a 50 ml centrifuge tube. Stopper and heat

the tube in a silicone oil bath at 90°C. for 1 hour. Cool the reaction mixture in an ice bath before proceeding to the following extraction step.

NOTE: Samples and standards may be stored overnight at room temperature in the propanol-sulfuric acid medium.

(d) Extraction of the Ester Derivative

Add 10 ml of water and 15 ml of n-hexane to the esterification mixture. Extract and centrifuge to clarify the n-hexane phase. Transfer the n-hexane extract to a 65 ml round bottom flask; reextract the aqueous-propanol phase with two additional 15 ml portions of n-hexane. Centrifuge after each extraction and combine the n-hexane extracts. (NOTE: Avoid taking any of the aqueous phase in this extraction step; otherwise, the n-hexane extracts will have to be washed with 3×10 ml of water and dried over sodium sulfate.) Concentrate this solution to 0.5 ml using a rotary evaporator equipped with a water aspirator and with a water bath set at 25° C. (NOTE: A rotary evaporator equipped with a water aspirator and with a vacuum pump and condenser may be used at this point.) Fortify this solution with 0.1 ml of acridine marker.

NOTE: Do not store the n-hexane extracts of the propyl ester derivative overnight. Continue to process these solutions by the following thin-layer chromatography step (e).

(e) Thin-layer Chromatography

(1) Quantitatively transfer the concentrated n-hexane extract to the "origin" of a 20 × 20 cm silica gel thin-layer plate, using a disposable pipette. When pipetting this extract, streak it in a uniform band approximately 15 cm across and approximately 20 mm above the lower edge of the plate, making sure not to scratch or remove appreciable portions of adsorbent and avoiding application of the sample to the sides of the plate. The applied band should not diffuse or penetrate to the end of the silica gel layer, but should remain 10 mm above the lower edge of the silica gel layer. Rinse the round bottom flask (containing residual n-hexane) with three portions of approximately 0.25 ml each of ethyl acetate; transfer each portion with the same pipette and cover the same area of the plate as described above. Following each application of the extract and ethyl acetate washes, evaporate the solvent from the plate by directing a stream of cool air to the sample zone ("origin"). Prior to chromatographic development, place an edge (approximately 5 mm deep) of the thin-layer plate into a tray of ethyl acetate so that the solvent will rise through the applied sample zone producing a narrow band approximately 10 mm above the "origin." Air dry this plate before chromatographic development.

(2) Place the prepared plate in a chromatographic chamber lined with blotting paper and saturated with the benzene-ethyl acetate system (85:15). Develop the plate twice in this system, maintaining straight solvent fronts and allowing the solvent front to reach the top of the plate during each irrigation. Air dry the thin-layer plate for approximately 5 minutes between the first and second irrigations. Each irrigation takes approximately 75 minutes. Developed plates should not be stored overnight. Examine the developed plate under long wavelength (365 nm) ultraviolet light and locate the blue fluorescent band of acridine (R_f , approximately 0.5). Mark out a 12 mm × 20 cm band of silica gel encompassing an area 5 mm above and 7 mm below the center of the acridine marker and extending from one side of the plate to the other.

NOTE: The relative mobilities of propyl quinoxaline-2-carboxylate and acridine must be checked in each laboratory to determine where a 12 mm × 20 cm zone of silica gel is to be exercised in order to quantitatively recover the propyl ester derivative. This may be accomplished by mixing 0.1 ml of acridine solution (1 mg/100 ml) with 0.4 ml of propyl quinoxaline-2-carboxylate (100 µg/ml) and chromatographing this solution as directed above. Examine the developed plate under long wavelength (365 nm) ultraviolet light and locate the blue fluorescent band of acridine (R_f approximately 0.5). Examination of the plate under short wavelength (254 nm) ultraviolet light locates the blue absorbing band of propyl quinoxaline-2-carboxylate (R_f approximately 0.5).

(3) Reduce the sample zone to a fine powder by making a series of horizontal cuts with a scalpel. Gently transfer this powder with the aid of a stainless steel spatula to glassine paper; pour this material into a burette funnel atop a small glass column packed with a glass wool plug. Elute the absorbent with ethyl acetate (about 6 ml), and collect the eluate to mark in a 5 ml volumetric flask. Examine this eluate by gas-liquid chromatography.

NOTE: Contamination of thin-layer chromatographic plates can be checked by gas-liquid chromatographic examination of an eluate prepared by processing a blank plate as in paragraph 1 above, starting at the point: "place an edge

(approximately 5 mm deep) of the thin-layer plate into a tray of ethyl acetate. . . ." If the plate is contaminated, examine alternate lots of precoated thin-layer plates.

(f) Standard Curve

Pipette 4 ml aliquots of quinoxaline-2-carboxylic acid working standard solutions C, D, and E, respectively, add 4 ml portions of 1 propanol-sulfuric acid reagent into 50 ml centrifuge tubes; stopper, react, extract, and concentrate as directed in the esterification and extraction steps described in subsections (c) and (d) above; however, omit the addition of acridine to the n-hexane concentrate and do not chromatograph it by thin-layer chromatography. Instead, reconstitute the n-hexane concentrate with ethyl acetate and quantitatively transfer this solution to a 5 ml volumetric flask to give working standard solutions C, D, and E. The final concentrations of working standard solutions C, D, and E, are 20, 30, and 40 ng/ml respectively, and are equivalent to 20, 30, and 40 ppb, respectively.

(g) Gas-liquid Chromatography

Separately inject 4 μ l of each of the working standard solutions C, D, and E, prepared as described above 5.014C(t) into the gas-liquid chromatograph to determine the retention time of propyl quinoxaline-2-carboxylate and the relative response of the EC detector. Construct a standard curve by plotting concentration (ppb) versus peak height (millimeters).

(NOTE: The reagent blank must show no interfering gas-liquid chromatographic peaks.) The peak height of propyl quinoxaline-2-carboxylate at the 30 ppb level (working standard solution D) should approximate 10 percent of full-scale deflection with a retention time of 5 minutes. Follow these injections with 4 μ l injections of the tissue eluates, allowing 20 minutes between injections to clear the instrument of background peaks.

Measure the peak heights of samples and determine their concentration (ppb) by reference to the standard curve.

(h) Calculations

From the standard curve and the observed peak height of quinoxaline-2-carboxylic acid in the sample, determine its concentration (ppb).

References:

- (1) Code of Federal Regulations, Title 21, §556.100.
- (2) Lynch, Martin J., Preparation and Evaluation of AG MP-50 Resin Columns for the Isolation of Quinoxaline-2-Carboxylic Acid: Pfizer, Inc., Groton, Ct. 06340, June 1, 1977.
- (3) Lynch Martin J., Rapid Determination of Quinoxaline-2-Carboxylic Acid in Swine Liver at 30 ppb by Ion-Exclusion and Electron Capture Gas-Liquid Chromatography; Pfizer, Inc., Groton, Ct. 06340, November 18, 1976..

5.015 Determination of Zeranol

This procedure is applicable to the determination of zeranol (6-(6,10-dihydroxyundecyl-beta-resorcylic) acid- μ -lactone) in tissue of cattle.

5.015A. Theory

A homogenized tissue sample (except fat) is lyophilized to a dry solid and extracted with methanol to remove the drug. The extract is hydrolyzed with acid and partitioned sequentially from chloroform to 1 N sodium hydroxide, to carbon tetrachloride, to 1 N sodium hydroxide, to ethyl ether, and dried to a residue. The residue is derivatized and quantitated by gas liquid chromatography using a flame ionization detector.

5.015B Apparatus

(a) Extraction assemblies, Soxhlet, improved, standard taper grindings, Pyrex brand glass, 1000 ml capacity, Sargent Catalog S-31265D, or equivalent.

(b) Flasks, freeze drying, wide mouth, 1000 ml capacity 24/40 standard taper grindings, Pyrex brand glass, Sargent Catalog S-28875-20-F, or equivalent.

(c) Flasks, homogenizing, 250 ml, Sargent Catalog S-61716, or equivalent.

(d) Funnels, separatory, Squibb stopper, with Teflon stopcock plug, Pyrex brand glass, 250 and 500 ml capacities, Sargent Catalog S-35815-20-F or G, or equivalent.

(e) Gas chromatograph, with flame ionization detector.

(f) Gas chromatography column: Glass, 6 feet by 1/4-inch packed with 3 percent by weight GE SE-52 (Applied Science Laboratories) deposited on P.E. celite 60-80 mesh, or equivalent. Condition the column by baking for 40-80 hours at 280°C with a helium flow, but detached from the detector input. Injections of 1-2 microliters of a 50/50 mixture of hexamethyldisilazane and trimethylchlorosilane will help remove active sites in the column.

(1) Prepare a TMS derivative of a 1,000- μ g zeranol standard as described in the procedure section. Inject 1- μ l quantities to determine whether the column is responding to the conditioning. After the column shows a response at the 1,000- μ g level, proceed to smaller quantities to optimize conditions.

(2) The column and chromatograph must be conditioned to achieve a minimum sensitivity response so that a peak 2 cm in height results from an injection of 5 μ l of standard preparation containing 1 μ g of zeranol in the derivative preparation. *This criterion must be met before tissue assay is attempted.*

(3) The column is brought to 250° C after conditioning and held at the temperature for at least 12 hours before making a run.

(g) Heating mantle, electric: Glas-Col, Sargent Catalog S-40866H, or equivalent.

(h) Hot plate: with gradient rheostat heat control, explosion proofed.

(i) Meat grinder: manually operated or equivalent.

(j) Steam bath.

(k) Syringe: Hamilton Micro-syringe Model 701, 10-microliter capacity, or equivalent.

(l) Balance: 0.1 sensitivity, 500 grams capacity.

- (m) Vials: 1-dram glass with plastic tops, Ownes-Illinois, Opticlear, or equivalent.
- (n) Virtis freeze drier: Sargent Catalog S-28881-80, or equivalent.
- (o) Virtis homogenizing mill, macro: Virtis No. 45, Sargent Catalog S-61700, or equivalent.

5.015C. Reagents

- (a) Carbon tetrachloride, N.F.: Fisher Scientific C-186, or equivalent.
- (b) Chloroform, N.F.: Fisher Scientific C-296, or equivalent.
- (c) Chromatograph gases: flow rates adjusted to maximize sensitivity for specific chromatograph.
 - (1) Carrier gas; conventional tank helium or nitrogen.
 - (2) Oxygen: conventional tank oxygen.
 - (3) Hydrogen: Linde high purity, or equivalent.
- (d) Column packing: 3 percent GE SE-52 (Applied Science Laboratories) on P.E. Celite 60-80 mesh (Johns Manville Product No. 154-0048), or equivalent.
- (e) Ether, anhydrous: Fisher Scientific E-138, or equivalent.
- (f) Hexamethyldisilazane: Dow-Corning, or equivalent.
- (g) Hydrochloric acid: analytical reagent grade.
- (h) Methanol: certified A.C.S., Spectranalyzed, Fisher Scientific A-408, or equivalent.
- (i) Phosphoric acid: analytical reagent grade
- (j) Pyridine: anhydrous, A.C.S. reagent grade.
- (k) Silylation reagent mixture: Pipet 8 ml each of pyridine and hexamethyldisilazane and 4 ml of trimethylchlorosilane into a clean glass vial with a polyethylene cap and mix thoroughly. Let stand overnight and decant supernatant liquid into a vial. Cap and store at room temperature for daily use. If kept dry, the reagent is stable for more than a month. Blanks are scanned by gas chromatography on each new bottle of (j), (f), and (n) material used in the silylation reagent mixture for possible peak interference in the region of zeranol derivative.
- (l) Sodium chloride: analytical reagent grade.
- (m) Sodium hydroxide: analytical reagent grade.
- (n) Trimethylchlorosilane: Dow-Corning, or equivalent.
- (o) Water: distilled in glass.
- (p) Zeranol: primary standard.
- (q) Solutions.
 - (1) 2N Hydrochloric acid in water.

(2) 3N Phosphoric acid in water.

(3) 2 percent (w/v) sodium chloride in water.

(4) 1N Sodium hydroxide in water.

(r) Standard Solutions.

(1) Stock solution A: Accurately weigh 0.1000 g of zeranol, primary standard, into a 250-ml beaker. Dissolve the standard in 80 ml of methanol and accurately dilute to 100 ml in a volumetric flask with methanol. The solution contains 1,000 μg per ml.

(2) Stock solution B: Dilute 10.0 ml of stock solution A to 100 ml with methanol to provide a standard containing 100 μg of the drug per ml.

(3) Stock solution C: Dilute 5.0 ml of stock solution B to 100 ml with methanol to provide a standard of 5 μg per ml.

(4) Stock solution D: Dilute 2.0 ml of stock solution B to 100 ml with methanol to provide a standard of 2 μg per ml. Transfer 1.0 ml of stock solution D to a 1-dram glass vial, evaporate to a dry residue in a vacuum desiccator at reduced pressure. The residue contains 2 μg of zeranol to be used as a calibration standard in operation of the gas chromatograph.

CAUTION: Apply vacuum slowly to avoid bumping.

5.015D. Determination

(a) Preparation of glassware: Glassware should be washed in detergent or chromic acid solution to remove contaminants and rinsed in water to remove traces of cleaning agents. Rinse with methanol before using.

(b) Preparation of sample:

(1) Collect muscle, liver, kidney, and tripe from a freshly sacrificed animal under the cleanest conditions possible.

(2) Grind the fresh tissue in a meat grinder, divide into 100-gram portions, and wrap in aluminum foil. Store wrapped tissue in a deep freeze. Fat should be wrapped in foil and stored in deep freeze. If sample is not to be stored proceed directly to step (7).

(3) Weight 100 g of partially thawed tissue into a 250-ml homogenizing flask, add 60 ml of water, and attach to a Virtis "45" Tissue Mill, or equivalent.

(4) Mix the materials at 45,000 rpm for 5 minutes to obtain a thin homogenate.

(5) Transfer the homogenate to a 1-liter, wide-mouth, freeze drying flask using 10-20 ml of water for a rinse.

(6) Place the flask on its side in a nearly horizontal position in a slurry of dry ice and acetone. Rotate the flask on its side as the homogenate cools to set down a uniform frozen solid layer on the wall of the flask.

(7) Mount the flask on a Virtis freeze drier, or equivalent, and lyophilize to dry solids. This operation usually requires 20-24 hours. (Stopping place.)

(8) Transfer the solid cake to a clean sheet of paper and crumble by hand to a size convenient for transfer to an extraction thimble.

(c) Extraction procedure for muscle, liver, kidney, and tripe.

(1) Transfer the solids to a single thickness 60×180 millimeters Soxhlet extraction thimble and compact the solids sufficiently to guarantee complete immersion during solid extraction.

(2) Transfer 600 ml of methanol to a 1-liter flask of a Soxhlet extraction assembly and place the thimble in the extractor. Mount a large glass funnel in the neck of the extractor with the stem extending into the thimble. Rinse the 1-liter freeze drying flask with three 50 ml portions of fresh methanol and transfer the rinses through the funnel into the thimble. Mount the condenser in the extractor and extract the solids for 15 hours. The extractor should be heated with the electric heating mantle so that a fill-empty cycle requires 18-24 minutes.

(3) Drain the methanol from the thimble. Composite the methanol from the extractor and flask in an 800-ml beaker.

(4) Rinse the flask with 10 ml of methanol and add to the methanol composite. Transfer 50 ml of 2N HCl down the flask side wall, and add to methanol composite. Concentrate to 125 ml by boiling on a hotplate.

(d) Extraction procedure for fat.

(1) Cut fat into $\frac{1}{4}$ -inch cubes. The lyophilization of fat is unnecessary since it is essentially water free.

(2) Transfer 100 g of the prepared fat to a 60×180 -ml extraction thimble and extract with 750 ml of methanol for 15 hours in the Soxhlet extractor. The extractor should be heated with the electric heating mantle so that a fill-empty cycle requires 18-24 minutes.

(3) Drain the methanol from the thimble. Composite the methanol from the extractor and flask in an 800-ml beaker.

(4) Rinse the flask with 10 ml of methanol and add to the methanol composite. Transfer 50 ml of 2N HCl down the flask side wall, and add to methanol composite. Concentrate to 125 ml by boiling on hotplate.

(e) Solvent partition.

(1) Transfer the methanol concentrate to a 500-ml separatory funnel, identified by number as 1. Rinse flask with 70 ml chloroform; add to separatory funnel and mix.

(2) Add 300 ml of water and without shaking allow liquid phases to separate.

(3) Withdraw the chloroform layer into a separatory funnel, identified by number as 2, containing 100 ml of 2 percent aqueous sodium chloride.

(4) Gently mix the contents of funnel 2 horizontally end to end 30 times and allow phases to separate. Usually about 20 minutes are required to obtain maximum chloroform separation.

(5) Withdraw the chloroform layer into a beaker.

(6) Extract with shaking the contents of funnels 1 and 2 successively with three more 50 ml portions of chloroform.

(7) Composite the chloroform extracts and concentrate to 125 ml by evaporation on a steam bath and cool to room temperature.

(8) Transfer the chloroform composite to a 250 ml separatory funnel, fitted with a Teflon stopcock, using 10 ml of chloroform as a rinse.

- (9) Extract the chloroform with three separate 20 ml portions of 1N sodium hydroxide solution retaining the emulsion in the sodium hydroxide phase. Agitation of sodium hydroxide with the chloroform extract for the first time is accompanied by the appearance of emulsion.
- (10) Perform an extraction by gently inverting the closed funnel and returning the funnel to an upright position.
- (11) Repeat phase mixing 30 times per extraction.
- (12) Allow phases to separate for 10 minutes. The time delay allows for gradual dissipation of the emulsion to improve phase separation. The zeranol transfers from the chloroform to the upper sodium hydroxide phase in this operation.
- (13) Composite the sodium hydroxide extracts.
- (14) Wash the sodium hydroxide extract with three 50 ml portions of chloroform using the technique as in step 9 and the same 10 minute interval for phase separation. Washing the chloroform removes the emulsion and unwanted-impurities from the sodium hydroxide phase.
- (15) Discard the chloroform washes. Transfer the sodium hydroxide extracts to a 250 ml beaker. Rinse each separatory funnel with two 5 ml portions of water and add to the sodium hydroxide extract. Wash each funnel twice with tap water and twice with distilled water before next use.
- (16) Neutralize the washed sodium hydroxide extract to pH 8.0 by dropwise addition of 3N phosphoric acid using a pH meter.
- (17) Transfer the pH 8.0 water extract to a 250 ml separatory funnel using 10 to 20 ml of water for a rinse.
- (18) Extract the solution with three separate 50 ml portions of carbon tetrachloride. The zeranol transfers to the lower carbon tetrachloride phase. Use the same 30 count phase-mixing technique as in step 11, and allow the mixture to stand 5 minutes for phase separation.
- (19) Composite the carbon tetrachloride extracts.
- (20) Extract the carbon tetrachloride composite with two 20 ml portions of 1N sodium hydroxide. Zeranol transfers from carbon tetrachloride to the upper sodium hydroxide phase. After phase mixing, allow the mixture to stand 5 minutes for phase separation.
- (21) Composite the sodium hydroxide extracts.
- (22) Wash the extract with two 50 ml portions of carbon tetrachloride. Allow the mixture to stand 5 minutes for phase separation. Discard the carbon tetrachloride washes.
- (23) Transfer the sodium hydroxide extract into a 250 ml beaker. Rinse the separatory funnel with two 5-ml portions of water and add to the sodium hydroxide extract. Wash each funnel twice with tap water and twice with distilled water before next use. Adjust the sodium hydroxide extract to a pH of 9.5 by dropwise addition of 3N phosphoric acid and transfer to a 250 ml separatory funnel using 10-20 ml of water for a rinse.
- (24) Extract the pH 9.5 water solution with three separate 30-ml portions of anhydrous ethyl ether. Allow the mixture to stand 5 minutes for phase separation. The zeranol transfers to the upper ether phase.
- (25) Composite the ether extracts in a 125 ml Erlenmeyer flask.
- (26) Reduce the volume of ether to about 1-2 ml by evaporation on a steambath.
- (27) Transfer ether residue to a 1 dram glass vial. Rinse down flask side wall with 1-2 ml of fresh ether and transfer to the glass vial.

(28) Continue evaporation of ether to 0.1 ml.

(29) Place vial in a vacuum desiccator and evaporate residue at line vacuum and room temperature overnight to dryness.

(30) Close vial with a plastic cap and submit ether residue for preparation of TMS derivative and gas chromatographic assay. (Stopping place.)

(f) Gas liquid chromatography.

(1) Start the gas chromatography and maintain the following suggested operational conditions:

Carrier gas pressure: 50 psi at tank.

Carrier gas flow rate: Sufficient to give zeranol derivative peak a retention time of 4-8 minutes.

Electrometer range: 10^2 or 10^1 .

Detector temperature: 10° C above column temperature.

Injection port temperature: 10° C above column temperature.

Column temperature: 230° - 270° C, operate isothermally.

Recorder sensitivity: 1 millivolt.

Recorder chart speed: 1 inch per minute.

Sample size: 1 microliter to 5 microliters as necessary to give desired peak area for quantitative measurement.

Septum: Replace each evening and allow to condition overnight at operational temperature.

Flame assembly: Remove silica ash from the flame assembly each week. The flame assembly is removed; the anode, flame jet, and chimney are cleaned with a nylon bristle brush. Water and acetone are drawn through the jet capillary to remove any foreign material.

(2) Add 0.2 ml to silylating reagent to the sample or to the zeranol standard.

(3) Stopper the vial and shake vigorously.

(4) Warm the vial at 40° - 50° C for a few minutes, then roll the vial on a horizontal plane to insure that all of the interior surfaces of the vial have been in contact with the reagent.

(5) Let vial stand for $\frac{1}{2}$ -hour in a warm area (40° C) to allow reaction to reach completion.

(6) Place vial in a small padded centrifuge tube and centrifuge to settle the precipitate and insure that all the liquid is at the bottom of the vial.

(7) Inject 1.0-5.0 μ l of clear solution into the chromatograph. At the beginning of the day's run, make 3-5 injections of a standard to condition the column for that day before taking quantitative data.

(8) Run known mixtures at the beginning, middle, and end of the day's run over the concentration range of samples to be analyzed to compensate for day-to-day sensitivity fluctuations and drift. If four or less samples are to be run, calibrating at the beginning and end of the run is sufficient.

5.015E. Calculations

Area values are obtained on known mixtures and samples by multiplying the net peak height by the peak width at half height or by counting squares. Area values obtained on knowns are plotted versus zeranor concentration. Calibration plots indicate a near linear function in the 0-10 µg range. Area values obtained on samples are converted directly to µg quantities using the curve. Control tests demonstrated a 70 percent recovery of zeranor from spiked wet beef liver and muscle necessitating a correction factor.

$$\text{Zeranor, parts per billion} = \frac{\text{Micrograms of zeranor found} \times 1,000}{W \times R}$$

Where:

R = Correction factor as determined in 5.015F.

W = Grams of tissue examined.

5.015F. Recovery Study

(a) Fortification of reagent blank.

(1) For those using this method for the first time either for recovery study or tissue assay, a solvent blank and solvent fortified with zeranor should be processed through the entire procedure. This preliminary operation will establish whether or not the procedure is free from contamination arising from solvents and glassware and demonstrate the level of recovery of the standard zeranor. Level of recovery should be in the same range as the samples.

(2) Transfer 600 ml of methanol to a 1-liter beaker. Add 50 ml of 2N HCl to the methanol and concentrate to 125 ml by boiling on a hotplate.

(3) Transfer 600 ml of methanol to a 1-liter beaker. Add 50 ml of 2N HCl to the methanol and concentrate to 125 ml by boiling on a hotplate. Spike the concentrate with 1.0 ml of stock solution D.

(4) Assay both samples as described in the procedure beginning extraction step 5.015D(e)1.

(b) Fortification of samples.

(1) Transfer 100 g portions of partially thawed tissues into 250 ml homogenizing flasks and set half of them aside to serve as tissue blanks.

(2) Add to the remaining samples 1 ml of stock solution D to serve as fortified samples to which 20 parts per billion zeranor have been added.

(3) Assay both fortified and unfortified tissue as described in the procedure section beginning with 5.015D(b)3.

Reference

Code of Federal Regulations Title 21 § 556.760.

5.016 Determination of Fenthion Residues in Animal Tissues

This procedure is applicable to the determination of Fenthion (0,0-Dimethyl 0-[4-(methylthio)-m-tolyl] phosphorothioate) and its metabolites in animal tissues.

5.016A. Theory

A weighed portion of tissue is ground in a mixture of acetone and chloroform and the aqueous phase is discarded. The organic phase is evaporated to dryness and the residue partitioned between petroleum ether and acetonitrile. The acetonitrile layer is evaporated to dryness and the residue is treated with an oxidation mixture and extracted with hydrochloric acid. The oxygen analog, sulfone, is extracted from the aqueous phase in chloroform and evaporated to dryness. The residue is dissolved in acetone and is quantitatively analyzed by means of a gas-liquid chromatograph using a KCl thermionic detector.

5.016B. Apparatus

- (a) Assorted laboratory glassware
- (b) Blenders: Waring or equivalent, equipped with one-quart jars.
- (c) Centrifuge: Damon/IEC Model BE-SO, or equivalent.
- (d) Food chopper: Hobart, or equivalent.
- (e) Gas chromatograph: equipped with a detector, or equivalent.
- (f) Rotary vacuum evaporator: all glass.

5.016C. Reagents

- (a) Acetone: reagent, A.C.S., redistilled.
- (b) Acetonitrile: technical, redistilled.
- (c) Chloroform: reagent, A.C.S., redistilled.
- (d) m-chloroperbenzoic acid, F.M.C. Corp., Villa Park, IL.
- (e) m-chloroperbenzoic acid reagent: 10 percent (w/v) in isopropyl ether. Observe warnings on container label. Prepare only the amount needed.
- (f) Fenthion Standard solution (5 $\mu\text{g}/\text{ml}$ in acetone):
 - (1) Weigh 0.05 g of Fenthion standard into a clean 100 ml volumetric flask. Make to volume with reagent acetone and shake to mix.
 - (2) Transfer 1 ml of this solution to a clean 100 ml volumetric flask. Make to volume with reagent acetone and shake to mix. This flask contains 5 $\mu\text{g}/\text{ml}$ of Fenthion.
- (g) Hydrochloric acid: 2.0N.
- (h) Hyflo Super-Cel: Johns-Manville.
- (i) Isopropyl ether: reagent ACS Fisher Cat. No. E-141.

- (j) Petroleum ether: redistilled.
- (k) Sodium hydroxide: analytical reagent, 0.5N.

5.016D. Determination

(a) *Preparation of Sample Material*

- (1) Grind the entire sample in a food chopper in the presence of an equal amount of dry ice.
- (2) Place the sample material in frozen storage overnight to allow the dry ice to sublime.

(b) *Extraction of Fat Tissue*

- (1) Weigh 25 g of the chopped sample into a blender jar.
- (2) Add 200 ml petroleum ether and blend for three minutes at high speed.
- (3) Filter, with suction, through a Whatman No. 42 filter paper covered with a 1/8-inch layer of Hyflo Super-Cel.
- (4) Transfer the filtrate to a 500 ml separatory funnel. (NOTE: If the filtrate becomes cloudy, warm the solution before the transfer is made.)
- (5) Return the filter cake and filter paper to the blender jar.
- (6) Add 200 ml of acetonitrile and blend for two minutes at high speed.
- (7) Filter, with suction, through a Whatman No. 42 filter paper into the same filter flask.
- (8) Transfer the filtrate to the separatory funnel containing the petroleum ether extract.
- (9) Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 500 ml separatory funnel containing 100 ml of Skellysolve B.
- (10) Shake the second separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a 1,000 ml round-bottom flask.
- (11) Repeat the extractions in Steps 9 and 10 using two 200 ml portions of acetonitrile.
- (12) Evaporate the combined acetonitrile extracts (600 ml) to dryness on the evaporator at 40° C Continue as in 5.016D(d).

(c) *Extraction of Other Tissues*

- (1) Weigh 50 g of the chopped sample into a blender jar.
- (2) Add 15 g of Hyflo Super-Cel and 200 ml of acetone and blend for three minutes at high speed.
- (3) Filter, with suction, through Whatman No. 42 filter paper.
- (4) Transfer the filtrate to a 1,000 ml separatory funnel.
- (5) Return the filter cake and filter paper to the blender jar.
- (6) Add 200 ml of chloroform and blend at high speed for three minutes.

(7) Filter, with suction, through a Whatman No. 42 filter paper covered with a 1/8-inch layer of Hyflo Super-Cel into the same flask.

(8) Rinse the blender jar with 100 ml of chloroform and add the rinsings to the dry filter cake.

(9) Transfer the filtrate to the separatory funnel containing the acetone extract.

(10) Shake the separatory funnel for 20 seconds, allow the layers to separate, and draw off the lower phase through a 32 cm Whatman No. 12 fluted filter paper into a 1,000 ml round-bottom flask.

(11) Evaporate the sample to dryness on the rotary evaporator at 40° C.

(12) Transfer the sample residue to a 500 ml separatory funnel with 200 ml of petroleum ether.

(13) Rinse the flask with 200 ml of acetonitrile and add the rinsings to the separatory funnel containing the petroleum ether solution.

(14) Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 500 ml separatory funnel containing 100 ml of petroleum ether.

(15) Shake the second separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a 100 ml round-bottom flask.

(16) Repeat the extractions in Steps 14 and 15 with two 100 ml portions of acetonitrile.

(17) Evaporate the combined acetonitrile extracts (400 ml) to dryness on the evaporator at 40° C.

(d) *Oxidation—All Samples* (Start an appropriate Fenthion standard)

(1) Dissolve the residue from the acetonitrile evaporation in 10 ml of the m-chloroperbenzoic acid reagent.

(2) Allow the sample to stand at room temperature for 30 minutes with occasional swirling. (NOTE: Do not allow the sample to remain in contact with the concentrated oxidant for more than 45 minutes.)

(3) Add 10 ml of isopropyl ether and transfer the sample to a 125 ml centrifuge separatory funnel.

(5) Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 125 ml centrifuge separatory funnel containing 20 ml of isopropyl ether.

(6) Add 80 ml of 2.0N hydrochloric acid to the first separatory funnel and shake for 30 seconds.

(7) Shake the second separatory funnel for 30 seconds, if necessary then centrifuge the sample for 5 minutes at 800 rpm.

(8) Draw off the lower phase into a 500 ml separatory funnel.

(9) Draw off the lower phase of the first 125 ml separatory funnel into the second 125 ml separatory funnel and repeat steps 7 and 8.

(10) Add 200 ml of chloroform to the 500 ml separatory funnel containing the aqueous extracts (160 ml).

(11) Shake the separatory funnel for 30 seconds, allow the layers to separate and draw off the lower phase into a second 500 ml separatory funnel.

(12) Repeat Step 11 with 100 ml of chloroform.

(13) Add 100 ml of 0.5N sodium hydroxide to the combined chloroform extracts and shake the separatory funnel for 30 seconds.

(14) Allow the layers to separate, and draw off the lower phase through a 32 cm Whatman No. 12 fluted filter paper into a 500 ml round-bottom flask.

(15) Evaporate the sample to dryness on the evaporator at 40°C, removing any traces of chloroform with a gentle stream of air.

5.016E. Gas Chromatographic Procedure

(a) Column: 16 inch \times 3 mm. id borosilicate glass column packed with 10 percent D.C. 200 and 0.2 percent QF-1, or equivalent on 80-100 mesh Gas Chrom Q.

(b) Carrier gas: Nitrogen, 35 ml/minute, 40 psi.

(c) Hydrogen flow: 35 ml/minute, 12 psi.

(d) Air flow: 425 ml/minute, 30 psi.

(e) Temperature: Column 210° C.
Injection port 225° C.
Detector 240° C.

(f) Recorder Chart speed: ½-inch per minute.

(gg) Electrometer range: 100 Attenuation-1

(1) Dissolve the residue from oxidation in 2 ml of acetone.

(2) Using a microliter syringe, inject 4 μ l of the sample or standard solution into the gas chromatograph.

(3) Identify the Fenthion peak by its retention time and measure the area produced on the recorder strip chart with a polar planimeter. At the operating conditions employed, the retention time for the Fenthion oxygen analog sulfone is 3.5 minutes.

5.016F. Calculations

Calculation of the ppm of Fenthion in a sample is done by use of the following equation in which response for an unknown is compared to the response for an amount of standard Fenthion carried through the procedure from the oxidation step. In the case of a 50 gram sample, 5 micrograms of standard Fenthion is used which corresponds to 0.1 ppm.

$$\text{ppm} = \frac{\text{Area (Sample)}}{\text{Area (Standard)}} \times \frac{\text{Attenuation (Sample)}}{\text{Attenuation (Standard)}} \times \frac{\text{Std. inj.}}{\text{Sample wt. in gms.}} \times \frac{\text{Dilution}}{\text{Factor}}$$

When using the aliquots and dilutions described in the above procedure, the equation simplifies to:

$$\text{ppm} = \frac{\text{Area (Sample)}}{\text{Area (Standard)}} \times \frac{\text{Attenuation (Sample)}}{\text{Attenuation (Standard)}} \times 0.1$$

Reference

R.J. Anderson *et al* Journal of Agriculture and Food Chemistry 14. 6, 619, (1961).

5.017 Polarographic Determination of Robenidine in Tissue

5.017A. Theory

Robenidine is extracted from the sample with ethyl acetate.* The filtered extract is evaporated and the residue partitioned between petroleum ether and 50 percent acetic acid. The acetic acid extract is extracted with chloroform and the residue from evaporation of the chloroform is taken up in 90 percent methanol and transferred to a CG-50 ion exchange column. After washing of the column with 90 percent methanol, the drug is eluted from the column with acidic aqueous methanol. Robenidine is detected in the column eluate by polarography and its concentration in the sample is determined by the standard addition technique.

5.017B. Apparatus

- (a) Polarograph - differential pulse or oscilloscope with a suitable X-Y recorder.
- (b) Explosion-proof blender with jars.
- (c) Rinco Vacuum Rotary Film Evaporator or equivalent with a dry ice-ethanol cold trap.
- (d) Chromatographic Columns: 250 mm length, 10.5 mm id equipped with a Teflon stopcock and 200 ml reservoir, Kontes Glass Company.
- (e) Centrifuge: Damon/IEC, Model V, Size #2 equipped with adapter, IEC No. 242, or equivalent.
- (f) Centrifuge Tubes: Separator, Plain, Teflon stopcock, Kimax (Kimble #4510F): or equivalent.
- (g) Assorted general laboratory glassware and equipment.

5.017C. Reagents

- (a) *Ethyl Acetate*: Reagent grade.
- (b) *Petroleum Ether*: Reagent grade.
- (c) *Chloroform*: Reagent grade.
- (d) *Acetone*: Reagent grade.
- (e) *Toluene*: Distilled in glass or equivalent.
- (f) *90 percent Methanol*: To 100 ml of distilled water add 900 ml of absolute methanol and mix.
- (g) *50 percent Acetic Acid*: To 500 ml of distilled water add 500 ml of glacial acetic acid and mix.
- (h) *Acidic Aqueous Methanol*: To 800 ml of 90 percent methanol in a 1000 ml volumetric flask add 16.6 ml of concentrated hydrochloric acid. Dilute the solution to 100 ml with 90 percent methanol and mix.
- (i) *Glassware Cleaning Solution*: Dissolve 30 g tetrasodium pyrophosphate and 3 g Alconox in 1 gallon of hot water.
- (j) *Mercury*: Purified, triple distilled.
- (k) *Amberlite CG-50 Ion Exchange Resin*: (H⁺ form), 100/200 mesh.

*In the case of blood, acetone is substituted for ethyl acetate.

(l) *Robenidine*: Purified grade, obtainable from the American Cyanamid Company.

(m) *Stock Solution*: Dissolve $0.100 \text{ g} \pm 0.0001 \text{ g}$ in 250 ml of absolute methanol and dilute to 500 ml with methanol. This solution contains $200 \text{ } \mu\text{g/ml}$. Store under refrigeration.

(n) *Solution A*: Pipet a 25 ml aliquot of the *stock solution* into a 200 ml volumetric flask and dilute to volume with methanol. This solution contains $25 \text{ } \mu\text{g/ml}$. Prepare this solution weekly. Store under refrigeration.

(o) *Nitrogen*: Oxygen free (containing less than 2 ppm oxygen).

5.017D. Determination

CAUTION: All glassware used in the procedure is cleaned with "Glassware Cleaning Solution," rinsed several times with deionized water and dried before use. Prior to use all glassware must be rinsed with methanol.

(a) *Extraction*

(1) Weigh $25 \pm 0.1 \text{ g}$ (W) of finely divided sample and transfer to a blender jar. In the case of blood, a 101 ml sample is used for the analysis. Run a recovery sample in parallel spiking the tissue with $100 \text{ } \mu\text{l}$ of standard solution A (0.1 ppm).

(2) Add 100 ml of ethyl acetate to the jar. In the case of fat, the sample is melted on a steam bath before the ethyl acetate is added. In the case of blood, 100 ml of acetone is substituted for the ethyl acetate.

(4) Blend for 5 minutes at moderate speed.

(5) Filter the contents of the jar through glass fiber filter paper into a 500 ml evaporation flask. Rinse the jar with two 10 ml portions of ethyl acetate and transfer the rinsings to the filter paper.

(6) Evaporate the contents of the flask on the Rinco vacuum evaporator at $35\text{-}40^\circ \text{C}$. In the case of blood, 25 ml of ethyl acetate is added to the flask in the last stages of the evaporation to help control foaming.

(7) After evaporation, add to the flask 25 ml of petroleum ether, swirl the flask to wet the inside surface and transfer the contents of the flask to a separator centrifuge tube.

(8) Rinse the flask with 50 ml of 50 percent acetic acid and transfer the rinsings to the centrifuge tube. Stopper the tube and shake its contents 1 minute.

(9) If required, centrifuge the tube 15 minutes at approximately 1,200 rpm.

(10) Draw off the lower (50 percent acetic acid) layer through glass fiber paper (contained in a funnel and prewet with 50 percent acetic acid) into a 500 ml separatory funnel.

(11) Rinse the evaporation flask with an additional 50 ml portion of 50 percent acetic acid and repeat the above operations beginning with step 8.

(12) Extract by 1-minute shakings the combined 50 percent acetic acid extracts in the 500 ml separatory funnel with successive 100- and 50-ml portions of chloroform.

(13) Transfer the chloroform extracts to a 500 ml evaporation flask.

(14) Evaporate the contents of the flask to ca 10 ml on a Rinco vacuum evaporator at 40°C , then add 10 ml of toluene to the flask and complete the evaporation to dryness. Add an additional 2 ml of toluene to the flask and again evaporate to dryness, repeat until the odor of acetic acid is no longer noticeable.

(b) *Cleanup*

(1) *Column Preparation*

Insert a glass wool plug in the column bottom. Transfer 2 g of CG-50 resin to a 50 ml beaker, add 30 ml of 90 percent methanol, swirl, allow to settle and pour off the supernatant liquid. Add a fresh 30 ml portion of 90 percent methanol to the beaker and repeat the above operations until the supernatant liquid is clear. Add a fresh 30 ml portion of 90 percent methanol to the beaker, swirl and with the column stopcock open, add the resin to the column. Rinse the column with several small portions of 90 percent methanol. Do not allow the volume of liquid to fall below the head of the column bed. Close the stopcock until used.

(2) *Elution*

Add 5 ml of 90 percent methanol to the residue in the flask and swirl so that the liquid wets the inside of the flask. Transfer, by disposable glass pipet, the contents of the flask onto the column. Rinse the flask with 10 ml of 90 percent methanol and transfer to the column as the original liquid reaches the head of the column. Rinse the flask with an additional 10 ml of 90 percent methanol and transfer to the column.

When the second rinsing reaches the head of the column, add 20 ml of 90 percent methanol directly to the column. When the last of the 20 ml reaches the head of the column, add 50 ml of acidic aqueous methanol to the column. In the case of blood, only 25 ml of acidic aqueous methanol is added to the column. Collect the first 25 ml of effluent (Va) in a 25 ml volumetric flask fitted with a disposable funnel. In the case of blood, only the first 10 ml of column effluent are collected.

5.017E. Polarographic Measurement

(a) *Polarographic setting for oscillopolarography*

(1) *Calibration*

Run a primary calibration of the polarograph for robenidine reduction potential and sensitivity as follows:

Pipet 4 ml of acidic aqueous methanol into a Davis cell containing 2 ml of mercury and add 20 μ l of the standard solution (25 μ g/ml) by means of a hypodermic syringe or micropipet. Place the cell in the polarograph bath and degas with nitrogen for five minutes. Set the instrument control as follows:

Starting Potential	-0.50
Normal or RC Derivative	50 milliseconds
Cond. Compensator	off
Slope Compensator	off
Shunt Scale Factor	1 \times 4
Amp. Scale Factor	\times 1

The polarographic trace obtained should show a summit potential at approximately -0.8 volts and response approximately 25 mm high on the oscilloscope screen. Response and summit potential may vary with the instrument and electrode used. (A slight shift in potential and suppression of the response is evident at times due to tissue background. Sensitivity is greatly affected by the electrode and it may be necessary to evaluate several before one with the proper sensitivity is found).

(2) *Sample Analysis for Oscillopolarography*

Transfer 4 ml (Vb) from the 25 ml volumetric flask (for blood, transfer from the 10 ml volumetric flask) to a polarographic cell containing approximately 2 ml of purified mercury. Outgas the sample for 5 minutes with nitrogen.

Using the previously described polarographic conditions and a X-Y recorder, record the polarographic trace.

(3) *Standard Addition*

Add accurately 20 μl (Vs) of the standard solution (25 $\mu\text{g/ml}$) (Cs) to the contents of the cell. Outgas the solution for 1 minute, then obtain the polarographic trace of the sample plus standard as described above. Add an additional 25 μl of standard solution and repeat the polarographic measurement. Proceed to calculations.

(b) Polarographic Measurement by Differential Pulse Polarography

(1) *Calibration*

Determine the Robenidine reduction potential and sensitivity as follows: Outgas the acidic aqueous methanol electrolyte solution in a gas washing bottle for 15 minutes. Fill the salt bridge tube with the electrolyte solution. Pipette 10 ml of the electrolyte into a polarographic cell. Connect the cell and outgas the solution for 3 minutes. Set the instrument controls as follows:

PAR Polarograph with dropping Mercury Electrode and X-Y Recorder

Reference electrode	Saturated Calomel-SCE
Mode	Differential Pulse
Modulation amplitude	50
Drop time	1 drop/sec.
Scan Rate	2 mv/sec.
Display direction	(+)
Scan direction	(-)
Initial potential	(-0.6 volts)
Range	(.75 volts)
Sensitivity	1 μA full scale

Scan the blank electrolyte solution. Add 40 μl of the standard robenidine solution (25 $\mu\text{l/ml}$) by means of a 100 μl syringe. Outgas the solution for 1 minute. Scan the solution. The polarogram obtained should show a summit potential at approximately -0.8 volts and a response approximately 40 mm high.

(2) *Sample Analysis*

Transfer 10 ml. (Vb) of the sample solution from the 25 ml (Va) volumetric flask to a polarographic cell. Outgas the solution for 3 minutes. Using the previously described conditions, scan the sample. If necessary, adjust sensitivity setting to bring peak within acceptable limits.

(3) *Standard Addition*

Accurately add sufficient amount (Vs) of Robenidine standard solution (25 $\mu\text{g/ml}$) (Cs) to the contents of the cell to give a response approximately twice that of the sample. Outgas the solution for 1 minute and repeat the measurement.

5.017F. Calculations

Measure the peak height (Po) of the sample and the peak height of the fortified sample (P). In the calculations use a fortified sample response whose peak height is close to twice the peak height of the unfortified sample.

$$\text{Robenidine} = \frac{P_o (V_s) (C_s) (V_a) (D.F.)}{P - P_o (W) (V_b) (R)}$$

Where:

P_0 = The measured value for the sample solution before addition of the robenidine standard solution.

P = The measured value for the sample after addition of the robenidine standard solution.

V_s = Volume of standard solution added to cell. (in ml)

C_s = Concentration of standard solution. (in $\mu\text{g/ml}$)

V_a = Volume of collected column effluent.

W = Weight of sample taken for analysis.

V_b = Volume of collected column effluent taken for analysis.

D.F. = Dilution factor—required when sample response is outside the linear range.

$$R = \text{Recovery factor} = \frac{\text{Amount of Robenidine found}}{\text{Amount of Robenidine added}}$$

Reference

Code of Federal Regulation, Title 21, § 556.580.

5.018 TLC—Densitometric Procedure for Sulfonamide Residues in Animal Tissues (STLC-F)

Note: This procedure supersedes the TLC-Densitometric Screening Procedure for Sulfamethazine in Swine Liver and Muscle.

5.018A. Introduction

A tissue residue assay for sulfonamides using thin layer chromatography and in-situ fluorimetric scanning for quantitation has been developed. After addition of an internal standard, sulfapyridine, tissue is extracted with ethyl acetate. The sulfonamides are then partitioned into glycine buffer (pH 12). The pH of the aqueous phase is adjusted to pH 5.25 and extracted with methylene chloride. The methylene chloride is evaporated to dryness and the residues reconstituted in methanol. Separation of the drugs from coextractives is carried out on silica gel plates containing a preadsorbent spotting layer. Visualization is accomplished by dipping in fluorescamine solution.

The procedure has been examined quantitatively for sulfamethazine, sulfadimethoxine, sulfaquinoxaline in swine, turkey and duck. The method has also been applied to the following drug/species combinations: sulfamethazine, sulfadimethoxine, sulfabromomethazine, sulfamethoxypyridazine, sulfaethoxypyridazine, and sulfachloropyridazine in beef and sulfathiazole in swine.

5.018B Apparatus

- (a) Farrand VIS-UV-2 chromatogram analyzer or equivalent
- (b) Tekmar Model SDT Tissumizer or equivalent
- (c) IEC PR 6000 centrifuge or equivalent (3000 rcf)
- (d) Temperature controlled heating strip
- (e) Whatman LK6D silica gel plates, 20 × 20 cm
- (f) pH meter
- (g) Vacuum aspirator with trap
- (h) 50 ml screw cap polypropylene centrifuge tubes (Corning #25330, Scientific Products #C3973-50)
- (i) 50 mv strip chart recorder
- (j) mechanical shaker, horizontal
- (k) oven
- (l) TLC developing tank (2)
- (m) stainless steel dipping tank or equivalent
- (n) Organomation N-Evap or equivalent device for evaporation by nitrogen and heat
- (o) Vortex test tube mixer
- (p) calculator capable of linear regression analysis
- (q) microliter syringes (100 to 100 ul)

- (r) UV light box
- (s) Styrofoam ice chest for insulating TLC tank

5.018C Reagents

- (a) Ethyl acetate: Distilled in glass or equivalent
- (b) Methylene chloride: Distilled in glass or equivalent
- (c) Chloroform: Distilled in glass or equivalent. No ethanol preservative added.
- (d) Tert-butanol: reagent grade
- (e) Methanol: Distilled in glass or equivalent
- (f) Acetone: Distilled in glass or equivalent
- (g) Concentrated hydrochloric acid (HCl), 1.0 reagent grade
- (h) 10N sodium hydroxide (NaOH) from reagent grade chemical.
- (i) fluorescamine dipping solution (30 mg in 250 ml acetone). Use for 8-9 plate developments. (Pierce Chemical Co.)
- (j) diethylamine, reagent grade
- (k) TLC solvent system — chloroform — tert-butanol (80:20 v/v). Prepare fresh daily.
- (l) 0.2M glycine (reagent grade) buffer — adjust to pH 12.25 with 10N NaOH. (Note: This procedure is written to be carried out at normal ambient temperatures of 68°C on a steam bath just prior to use.)
- (m) 2M phosphate buffer — mix 2M potassium hydrogen phosphate with 2M potassium dihydrogen phosphate such that pH = 5.25
- (n) 1.7M HCl/2M phosphate buffer, pH 5.25, 1:1, v/v)
- (o) 0.20M Phosphate buffer — Weigh 45.646g $K_2HPO_4 \cdot 3H_2O$ and dissolve in 1000 ml distilled water (solution 1). Weigh 27.218g KH_2PO_4 and dissolve in 1000 ml distilled water (solution 2). Adjust solution 1 with pH meter to $7.55pH \pm 0.05pH$, with solution 2. (Approximately 80 ml solution 1 plus 20 ml solution 2 will result in 100 ml of 7.6 pH solution.)

5.018D Preparation of Stock Standards — 1 mg/ml

- (a) Stock solutions of the internal standard, (I.S.), sulfapyridine, and the sulfonamides of interest are prepared as follows: Weigh accurately 100 mg of each sulfonamide into separate 100 ml volumetric flasks. Dissolve and bring to volume with acetone. Store in polyethylene bottles at -10°C. Prepare new stock solutions monthly.

5.018E. Preparation of Working Standards

- (a) Fortification Standards — Prepare fresh weekly
 - (1) 10 ug/ml Standards

Into a 100 ml volumetric flask, pipette 1 ml of each sulfonamide of interest (analyte(s)) stock solution. Do not add internal standard. Into a separate 200 ml volumetric flask pipette 2 ml of the I.S. stock solution. Bring with 0.20 M phosphate buffer, pH 7.6. Store in polyethylene bottles and refrigerate.

(2) Solution D (2.50 ug/ml I.S.)

Into a 100 ml volumetric flask, pipette 25 ml of the 10 ug/ml I.S. solution. Bring to volume with 0.20 M phosphate buffer, pH 7.6 to obtain Solution D (2.50 ug/ml I.S.). Store in a polyethylene bottle and refrigerate.

(3) Solution C (5.00 ug/ml sulfonamide of interest and 2.50 ug/ml I.S.)

Into a 100 ml volumetric flask, pipette 25 ml of the 10 ug/ml I.S. and 50 ml of the 10 ug/ml standard of the sulfonamide or mixed sulfonamides of interest and bring to volume with 0.20 M phosphate buffer, pH 7.6. Store in a polyethylene bottle and refrigerate.

(4) Solution B (2.50 ug/ml sulfonamide of interest and 2.50 ug/ml I.S.)

Into a 50 ml volumetric flask, pipette 25 ml of Solution C and bring to volume with Solution D. Store in a polyethylene bottle and refrigerate.

(5) Solution A (1.25 ug/ml sulfonamide of interest and 2.50 ug/ml I.S.)

Into a 50 ml volumetric flask, pipette 25 ml of Solution B and bring to volume with Solution D. Store in a polyethylene bottle and refrigerate.

5.018F. Determination

(a) Extraction

(1) Weigh 2.50 g of frozen preground tissue into a 50 ml polypropylene centrifuge tube. Allow to thaw. Select a blank tissue and fortify three samples of this tissue with 100 ul aliquote of standards A, B, and C, respectively with the SMI microrepettor.

(2) Add 100 ul of standard D to all incurred samples.

(3) Allow samples to stand for 15 minutes.

(4) (a) Liver — Add 25 (\pm 1.0) ml of ethyl acetate via repipet. Do not tissumize.

(b) Muscle — Add 25 ml of ethyl acetate via repipet and blend for 60 seconds with tissumizer. Scrape any adhering tissue off tissumizer into the centrifuge tube.

(c) Both tissues — Seal tube with screw cap. Shake once or twice by hand to check for leakage.

(5) (a) Liver — Shake on horizontal shaker for 20 minutes at approximately 250 cycles/minute.

(b) Muscle — Proceed to (6).

(6) Centrifuge 5 minutes at 2500 rpm.

(7) Decant supernatant into a clean 50 ml polypropylene centrifuge tube and add 10 ml of 0.2M glycine buffer. Discard tissue residue and original tube.

(8) Shake on mechanical shaker for 5 minutes.

(9) Centrifuge 5 minutes at 2500 rpm.

(10) Remove organic phase (upper layer) with Pasteur pipette attached to vacuum aspirator. Take care to remove any solid or emulsified material remaining at interface or clinging to tube wall.

(11) Adjust pH to 5.25 (± 0.10) by adding 2 ml of 1:1 1.7M HCl/2M phosphate buffer (pH 5.25). Check pH and adjust if necessary with additional buffer or 1.0N NaOH or 1.0N HCl.

(12) Add 5 ml hexane and shake on mechanical shaker for 5 minutes.

(13) Centrifuge 5 minutes at 2500 rpm.

(14) Remove organic phase (upper layer) with Pasteur pipette attached to vacuum aspirator. Use gentle vacuum to aspirate upper layer, especially if solids are present at the layer interface.

(15) Add 10 ml methylene chloride and shake on mechanical shaker for 5 minutes.

(16) If no emulsions are present, centrifuge 5 minutes at 2500 rpm. If emulsions are present, centrifuge 10 minutes at 3500 rpm.

(17) Aspirate aqueous phase (upper layer). Entire layer can be completely removed by tilting tube and keeping pipette aspirator at tube wall. Take care to remove any fat or particulates at the interface. If a gelatinous plug is present between the aqueous and organic phases, do not remove or aspirate it or poor recovery will result.

(18) Add 10 μ l of diethylamine to the methylene chloride phase in the tube just prior to evaporation.

(19) Evaporate contents of the centrifuge tube under a stream of nitrogen on a N-Evap (40°C) just to dryness. Do not allow residue to dry out.

(20) After the level of methylene chloride has declined to 5 ml, rinse the sides of the tube with an aliquot (ca 2 ml) of methylene chloride. Repeat rinse when volume declines to approximately 2.5 ml and again at approximately 1.0-1.5 ml.

(21) Dissolve residue in methanol (100 μ l) and vortex samples for 30 seconds. Allow to stand for 5 minutes so that insoluble oils settle to bottom of tube.

(22) Keep tubes tightly stoppered in case an additional analysis is required.

(b) Thin Layer Chromatography

(1) Using a micropipette, spot 20 μ l of each fortified control sample and up to 14 incurred tissue samples on the preadsorbent layer of the LK6D plate with the preadsorbent heated to 85°C on an AIS spotter or equivalent heat strip. Take care not to take up any of the insoluble oil fraction as this distorts the chromatography. A clean, oil-free 20 μ l aliquot can be removed by tilting the tube so that the less viscous methanol separates from the oily residue. Position the spot approximately 1.0-1.5 cm from the preadsorbent silica gel interface. Locate the three standard spots across the plate as follows: Position the 0.05 ppm standard exactly in the center of the plate. Plate the 0.10 and 0.20 ppm standards 1-2 lanes in from the outermost samples, i.e. empty lane, sample 1, 0.10 ppm standard, sample 2, sample 3 . . . sample n-1, 0.20 ppm standard, sample n, empty lane. Do not spot on the outermost lanes of the plate as edge effects can distort both band shape and R_f , leading to inaccurate quantitation. Note: For densitometry, the spot must be applied in one even application such that the expanding spot just reaches the lane edges at the end of the application.

(2) Develop the plate 1 cm from the interface in methanol. Remove and dry for 1 minute in an oven at 100°C.

(3) Develop the plate 6 cm from the interface in chloroform-tert-butanol (80:20) in a saturated tank contained in an insulated ice chest.* Dry plate for 1 minute in an oven at 100°C to insure consistent R_f values across the plate. If

*See Appendix

multiple sulfonamides are suspected, carry out separation at 29-32°C. This can be accomplished by placing the tank in a forced air oven with heating elements off, but with the blower on.

(4) Dry plate in oven for 1 minute at 100°C and dip the plate in the fluorescamine solution. Keep plate in the solution only 1-2 seconds after fully submerged, otherwise background color will be excessive. Replace fluorescamine solution after 8-9 individual dippings.

(5) Allow plate to develop in the dark at room temperature for 15-30 minutes and view under UV light.

(6) Scan on densitometer using 410 nm for excitation and emission wavelengths, respectively. Lanes that contain a visible sulfapyridine band (indicating recovery of the I.S.) but no visible incurred sulfonamide bands need not be scanned. Sample lanes without a visible internal standard band (indicating low recovery) should be scanned, as visual estimation in such cases is not reliable.

(7) Scan an unused lane to determine plate background for baseline correction.

(c) Calculations

(1) For each plate developed, construct a standard curve from the three fortified samples applied to that plate as follows:

(a) Measure the peak heights of the sulfonamide(s) of interest and the internal standard peaks of each fortified sample and calculate their respective peak height ratios.

$$\frac{\text{sulfonamide of interest peak height}}{\text{internal standard peak height}}$$

(b) Using linear regression, construct a standard curve of sulfonamide concentration vs. peak height ratio.

The equation is: $y = mx + b$

where x = Sulfonamide/I.S. peak height ratio

y = sulfonamide concentration (ppm)

m = slope

b = y intercept

The correlation coefficient (r) should be ≥ 0.995

(2) Calculate the standard error of estimate (S_{y-x}) using the formula:

$$S_{y-x} = \sqrt{\frac{\sum y^2 - \frac{(\sum xy)^2}{\sum x^2}}{n - 2}}$$

where n = # of standard points

The standard error should be ≤ 0.02 .

(3) Action level for confirmation = 0.11 – 0.15 ppm

(4) Using the regression slope and intercept, compute the sulfonamide concentration (y) for each incurred sample from the measured peak height ratio.

APPENDIX

Stopping Points

Within-day — Essentially any step can be used as a stopping point during the course of the day although it is not desirable to allow the sulfonamides to remain in the strongly basic glycine buffer for more than one hour. Likewise, care should be exercised during the concentration to dryness step not to allow this step to go unattended. Significant losses due to adsorption may occur if the residue is allowed to evaporate beyond the "just to dryness" stage and remain that way for any length of time while heated.

Overnight — In addition to the above mentioned exceptions, do not dip the developed plate in fluorescamine unless it is scanned the same day. Unacceptable diminution of fluorescence will occur overnight at room temperature. This can be overcome by storing the derivatized plate overnight in a sealed plastic bag at -10°C . Quantitation from plates stored in this manner is acceptable. One can, however, successfully spot one day and develop the next, or develop one day and dip the plate the next day.

Instrumentation

Although most densitometers capable of fluorescence detection can be used for the procedure, experience has shown that certain instrument configurations are superior in terms of baseline drift, noise, etc.

In order of decreasing desirability:

- double monochromator system (on both exciter and emission light paths)
- single monochromator system (on excitation source) and interference or sharp cutoff filter (on emission side)
- interference filter (400 nm on excitation source) and sharp cut-off (K460 nm filter on emission side)

Slit width should be adjusted to be as close as possible to full lane width (ca. 8 mm on a LK6D plate). This approach minimizes errors due to sample distribution errors across the band width from spotting technique.

TLC Solvent Systems

Sulfathiazole can be distinguished from sulfapyridine using the chloroform-tert-butanol solvent system, but unless conditions are optimal, densitometric resolution is incomplete. For this reason if sulfathiazole is present or suspected, the chloroform-tert-butanol solvent system (80:20) should be washed with water prior to use. The increase in polarity from the trace water absorbed by the organic phase will substantially increase the separation between sulfathiazole, sulfapyridine and sulfamethazine. However, the resolution between sulfamethazine, sulfaquinoxaline and sulfadimethoxine will decrease. The resolution between all adjacent bands can be modestly increased in low humidity conditions by equilibrating the plate prior to development in a tank containing several beakers of sulfuric acid — water (50:50).

5.019 GC/EI-MS Detection/Confirmation of Sulfonamide Residues in Liver and Muscle Tissue of Swine, Poultry and Cattle.

5.019A. Theory

Sulfonamides are extracted from tissue utilizing a modification of the Tishler Method A procedure, followed by GC/EI-MS detection and confirmation.

The GC/EI-MS detection/confirmation procedure is based on the technique of selected ion monitoring (SIM). For this analysis three ions are monitored. The ions monitored are dependent on two specific sulfonamides being assayed. The ion current from each of the ions monitored is accumulated throughout the GC run and stored on magnetic tape. At the end of the GC run, ion current vs time for all ions is plotted. Peaks appearing in the ion current profiles are identified. The retention times and areas for each peak are calculated and reported. The identity of a specific sulfonamide is confirmed by the presence of the ions appearing at the proper GC retention time and present in the proper relative abundances.

5.019B. Apparatus

1. 24 cm Whatman 2V fluted filter paper
2. Rinco vacuum rotary film evaporator, Model 1007-4, or equivalent
3. 125 ml and 250 ml separatory funnels
4. 100 ml beakers
5. pH meter — Corning Model 125, or equivalent
6. Glass-stoppered graduated 15 ml centrifuge tubes
7. Diazomethane generator: Aldrich Chemical Co. (No. Z10, 159-1), or equivalent.
8. Vortex test tube mixer — Vari Whirlmixer — VWR Scientific, or equivalent.
9. Virtis blender — Model 45 — Virtis Co., or equivalent
10. 500 ml Virtis flasks
11. 15 cm and 6 cm glass funnels
12. 1000 ml round bottom flasks (heavy duty)
13. 9 cm Whatman No. 42 filter paper
14. N-Evap, Model 111 — Organomation Assoc. Inc., or equivalent
15. Nitrogen (High Purity)
16. Hewlett Packard 5992 GC/MS, or equivalent, equipped with a silicon membrane separator — GC column: 2 mm ID × 3 ft glass packed with 3% OV-17 on 80/100 mesh Gas Chrom Q.

5.019C. Reagents

1. Acetone: Distilled in glass, or equivalent
2. Concentrated hydrochloric acid (HCl): reagent grade

3. 5N HCl in distilled water
4. 1N HCl in distilled water
5. Trisodium citrate solution: Add 720 g reagent grade trisodium citrate dihydrate to one liter of distilled water
6. Ethyl ether: Distilled in glass, or equivalent (peroxide-free)
7. 10N Sodim Hydroxide
8. 3N Sodium Hydroxide
9. Methylene chloride (MeCl_2): Distilled in glass, or equivalent
10. Anhydrous methanol: Distilled in glass, or equivalent. Make anhydrous by shaking with and storing over anhydrous sodium sulfate.
11. Chloroform: Distilled in glass, or equivalent (no preservative)
12. Diazomethane: Prepare using manufacturer's instructions and the following reagents:

NOTE: Diazomethane preparation must be carried out in a hood behind a protective screen or shield. Operator should wear gloves to prevent skin contact with reagents or diazomethane. Caution must be observed when handling diazomethane as it is toxic and under some conditions explosive. The recommended conditions and total volume of diazomethane generated minimize the instability of the compound. Freshly made diazomethane solution is golden yellow in color and can be used for a maximum of 1 week. Store in freezer.

- a. N-methyl-N-nitroso-p-toluenesulfonamide (PTS): reagent grade. Handle with caution.
- b. Ethyl ether: anhydrous reagent grade.

NOTE: Ethyl ether added to collection vessel must be anhydrous. Traces of water cause decolorization of diazomethane solution with accompanying poor derivatization.

- c. Diethyleneglycol-monoethyl ether (Carbitol): reagent grade
 - d. Potassium hydroxide (KOH) 60% (W/V) aqueous, reagent grade
13. Hexane: Distilled in glass, or equivalent
 14. Ethyl acetate: Distilled in glass, or equivalent.
 15. Diethylamine (DEA): Reagent grade, Fisher Scientific
 16. Pentafluoropropionic anhydride (PFPA): Pierce Chemical Company

5.019D Standard Solutions

Sulfonamide stock solution: 50 ug per ml in MeOH of SMZ, SDM, SQX, STH and SBM.

5.019E. GC/EI-MS Quantitation/Confirmation

1. Tishler Option A

- a. Weigh 50.0 g (± 0.1 g) ground frozen tissue into a 500 ml Virtis flask. Select a blank tissue as a control. A second blank tissue sample is spiked at the 0.1 ppm level with sulfonamide stock solution (100 μ l).
- b. Add 100 ml of 1:1 chloroform; acetone to the flask.
- c. Blend for 1 minute at low speed with a Virtis blender.
- d. Decant and filter (no vacuum) the liquid through a 24 cm Whatman 2V fluted filter paper into a 1000 ml round bottom flask. Use a small spoon to retain the tissue in the Virtis flask while the solvent is being decanted. Filtrate must not contain any particulate matter. Filtrate must be clear and not cloudy. Allow filtrate to drain before adding extract from next step.
- e. Repeat steps (b, c, and d) two additional times. Transfer all tissues to the filter paper after the third extraction.
- f. Rinse flask from step (a) with about 25 ml 1:1 chloroform: acetone, transfer the rinsings to the filter paper, and allow to drain. Rinse filter paper and contents with three equal aliquots of 1:1 chloroform:acetone, totaling 60 ml, allowing filter paper to drain between aliquots. If combined filtrates are not clear, refilter and rinse filter paper with about 20 ml 1:1 chloroform:acetone.
- g. Evaporate sample extract on a rotary evaporator at 55°C ($\pm 5^\circ\text{C}$) to an oily residue (1-2 ml). Remove from rotary evaporator promptly. Time for evaporation should not exceed 25 minutes.
- h. Quantitatively transfer the residue to a 250 ml separatory funnel using, in the order listed, four 25 ml portions of hexane, two 3 ml portions of acetone and two 25 ml portions of hexane.
- i. Add 10 ml of 1 N HCl to the 250 ml separatory funnel. Shake gently for 2 minutes and allow phases to separate. Be certain to obtain good contact between phases in order to remove all fat from aqueous phase. Emulsions may be eliminated by placing separatory funnel in a water bath (60°C). If any sample within a set requires a water bath treatment to break an emulsion then all samples within that set should be so treated.
- j. Filter aqueous phase through a 9 cm Whatman No. 42 filter paper into a 125 ml separatory funnel. Repeat the extraction three times with 5 ml portions of 1N HCl, drawing off the acid phase and combining filtrates in the 125 ml separatory funnel.
- k. Wash the filter paper with three 1 ml portions of 1N HCl.
- l. Add 3.0 ml of 10N NaOH to the 125 ml separatory funnel and mix. Determine the pH: if it is not 12-13, add additional 10N NaOH with mixing to attain this pH.
- m. Shake the aqueous basic solution for one minute with a 25 ml portion of chloroform (CHCl_3). Allow phases to separate completely, discard CHCl_3 . Repeat CHCl_3 extraction a second time discarding CHCl_3 . Quantitatively transfer aqueous phase to a small beaker (~ 100 ml).
- n. Buffer by adding 25 ml of saturated trisodium citrate.
- o. Adjust the pH with a pH meter to 5.25 ± 0.10 by adding NaOH or HCl as required.
- p. Quantitatively transfer contents of beaker to a 125 ml separatory funnel, add 15 ml of MeCl_2 and shake for 1 minute.
- q. Allow phases to separate and transfer the MeCl_2 to a 50 ml conical centrifuge tube.

r. Repeat MeCl_2 extraction two times. Recheck the pH of the aqueous phase after the first extraction and readjust to 5.25 ± 0.1 if necessary.

s. Evaporate the contents of the centrifuge tube to dryness under a stream of nitrogen on N-Evap at 45°C .

t. Dissolve the residue in 2 ml anhydrous methanol.

u. Add 2 ml hexane and mix for 30 seconds with a vortex mixer.

v. Allow phases to separate, discard hexane layer (top).

2. Derivatization

NOTE: Prior to derivatization of the sample workup, analysts should verify that the derivatization process is functioning. This can most easily be accomplished by preparation of a reagent standard as described in section 5.019E 3a-h.

a. Evaporate sample extract to approximately 1 ml.

b. Add 1 ml of diazomethane solution, mix with a vortex mixer and allow to stand at room temperature for 5 minutes.

c. Transfer solution from 2b. into a 15 ml or smaller concentrator tube and evaporate to dryness under a stream of nitrogen on N-Evap at 45°C .

d. Dissolve methylated residue in 200 μl of EtOAc and mix.

e. Add 100 μl freshly prepared 20% (v/v) PFPA in hexane.

f. Add 100 μl 10% (v/v) DEA in EtOAc and allow to stand 15-20 minutes.

g. Evaporate to dryness under a stream of nitrogen on an N-Evap at 45°C .

h. Reconstitute sample residue in 200 — 400 μl EtOAc.

NOTE: Sample is now ready for GC/EI-MS analysis.

3. Preparation of Standard. (0.1 ppm)

a. Add 100 μl of sulfonamide stock solution (50 $\mu\text{g}/\text{ml}$) to a 15 ml concentrator tube.

b. Add 1 ml of freshly prepared diazomethane solution and allow to stand at room temperature for 5 minutes.

c. Evaporate to dryness under a stream of nitrogen on N-EVAP at 45°C .

d. Reconstitute methylated residue in 200 μl of EtOAc and mix on vortex mixer.

e. Add 100 μl of a freshly prepared 20% solution of PFPA in hexane.

f. Add 100 μl of a 10% solution of DEA in EtOAc and allow to stand for 15 to 20 minutes.

g. Gently evaporate to dryness under a stream of nitrogen on an N-EVAP at 45°C .

h. Reconstitute in 200 μl — 400 μl EtOAc.

NOTE: Standards prepared in this manner should be stable for 2-3 weeks provided they are stored at room temperature and protected from ultraviolet light.

4. GC parameters

a. GC parameters

- (1) Initial column temp: 210°C
- (2) Time at temp 1: 0 min.
- (3) Injector temp: 260°C
- (4) Final temp: 260°C
- (5) Program rate: 5°C/min
- (6) Time at temp 2: 10 min
- (7) Total run time: 21.5 min
- (8) Helium flow rate: 30 ml/min \pm 0.5 ml

NOTE: Using the conditions above, the elution order and approximate retention times for the five sulfonamides studied were:

Sulfathiazole	4.0 minutes
Sulfamethazine	5.0 minutes
Sulfadimethoxine	7.0 minutes
Sulfabromomethazine	7.5 minutes
Sulfaquinoxaline	8.5 minutes

b. Mass spectrometer parameters

(1) Mass analyzer and ion optics areas are set according to parameters determined by an autotune program. An autotune must be run prior to sample analysis.

(2) Parameters necessary for select ion monitoring (SIM) for specific sulfonamides are listed below:

Sulfamethazine:

Ion	Dwell time	Identification
373	500 msec	M-65
374	500 msec	M-64
238	500 msec	M-200

Sulfadimethoxine:

Ion	Dwell time	Identification
405	500 msec	M-65
406	500 msec	M-64
238	500 msec	M-232

Sulfaquinoxaline:

Ion	Dwell time	Identification
395	500 msec	M-65
396	500 msec	M-64
238	500 msec	M-222

Sulfathiazole:

Ion	Dwell time	Identification
350	500 msec	M-65
351	500 msec	M-64
238	500 msec	M-177

Sulfabromomethazine:

Ion	Dwell time	Identification
451	500 msec	M-65
452	500 msec	M-64
238	500 msec	M-278

c. Select the appropriate mass spectrometry parameters and conduct the setup routine for SIM data acquisition.

d. Inject 2.0 ul to 4.0 ul of the previously prepared reagent standard and plot a reconstructed ion current profile at the end of the run.

e. Inject 2.0 ul to 4.0 ul of each sample and plot a reconstructed ion current profile at the end of each run.

f. Compare the retention time of the sample with the retention time of the standard. In addition establish that the M-65/M-64 ion ratio for the sample and standard are within $\pm 20\%$ of each other. If these conditions are met the drug can be considered to be confirmed.

5.020 One Trap Mineral Oil-Vacuum Distillation-TEA Nitrosamine Procedure for Bacon (Prefried & Ground)

5.020A. Theory

This procedure revolves around the Thermal Energy Analyzer, a device manufactured by Thermo Electron Corporation that provides detection of nitrosamine compounds.

A weighed sample of approximately 25 g is placed in a 500 ml flask and covered with mineral oil. The flask is connected, through a trap in liquid nitrogen, to a vacuum system. Under vacuum, the flask is heated slowly to 120°C and the volatile material is trapped. See Figure 1.

The trap and adapter are rinsed with methylene chloride. The washings and aqueous distillate are collected in a separatory funnel. The distillate is extracted several times. The combined extracts are dried with anhydrous sodium sulfate.

Under controlled conditions, the extract is concentrated to 1.0 ml. Suitable standard solutions are injected into the gas chromatograph attached to the TEA. The sample extract is injected and the readout is compared to the readout from the standard solution injection.

5.020B. Apparatus

Suppliers listed are those convenient to the FSIS laboratories, and the items listed are those found to be suitable for the analyses. Endorsement advocacy of these suppliers or their products is neither expressed nor implied.

Names and addresses of the suppliers mentioned are listed below.

Kontes Glass Co., Inc.
100 Spruce Street
Vineland, NJ 08360

Arthur H. Thomas Co.
Vine Street at Third
P.O. Box 779
Philadelphia, PA 19105

Curtin Matheson Scientific Co.
2511 46th Street
Indianapolis, IN 46205

Kimble Products
Division of Owens-Illinois, Inc.
P.O. Box 1035
Toledo, OH 43666

Cole Parmer Instrument Co.
7425 North Oak Park Avenue
Chicago, IL 60648

GCA/Precision Scientific
3737 West Cortland Street
Chicago, IL 60647

Organomation Associates, Inc.
10 C Bear Foot Road
Northborough, MA 01532

Shimadzu Scientific Instruments, Inc.
Oakland Ridge Industrial Center
Columbia, MD 21045

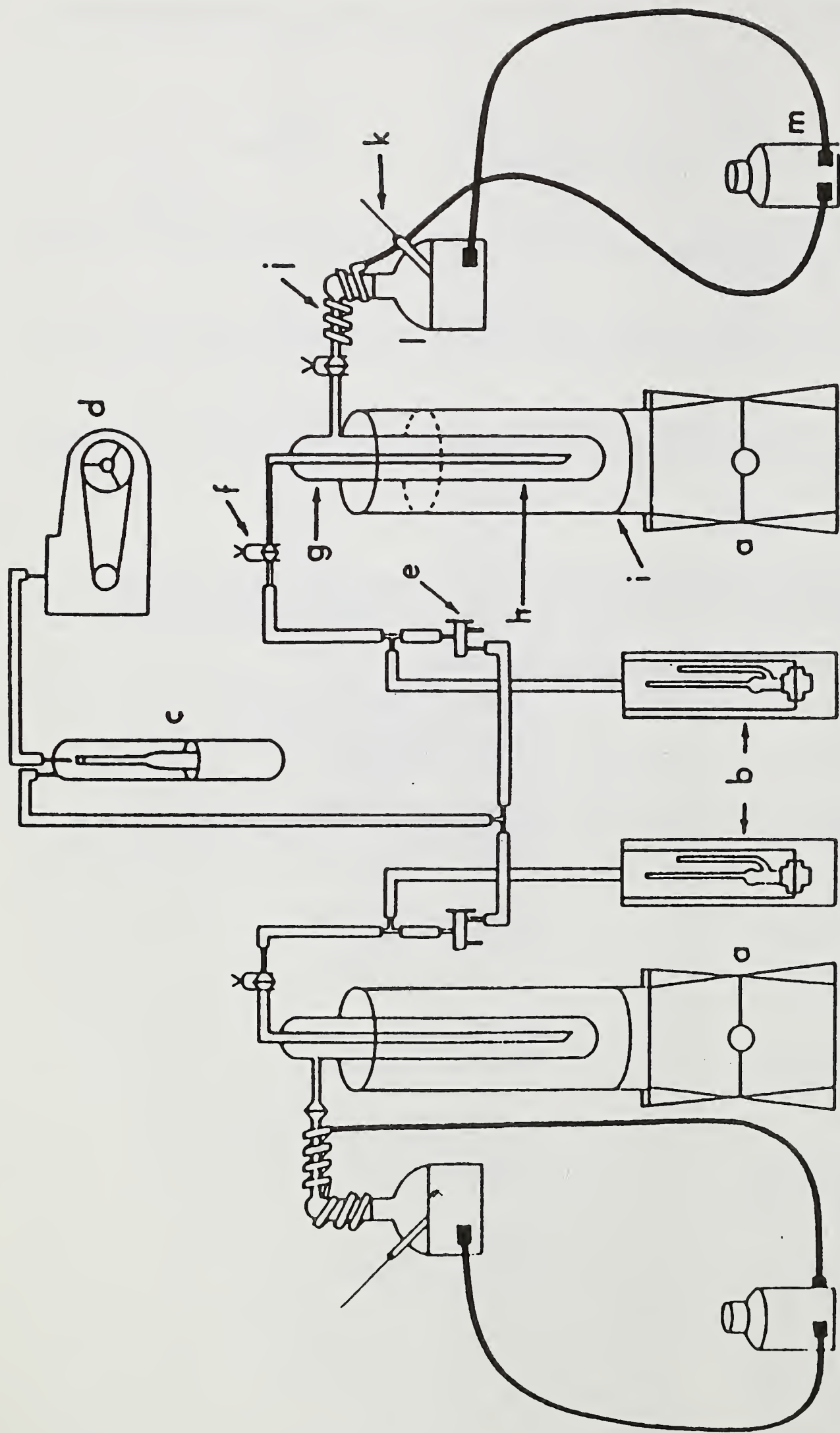
Thermo Electron Corporation
85 First Avenue
Waltham, MA 02154

Roger Gilmont Instruments
159 Great Neck Road
Great Neck, NY 11020

Fisher Scientific Co.
7722 Fenton Street
Silver Spring, MD 20910

Burdick & Jackson Laboratories, Inc.
1955 South Harvey Street
Muskegon, MI 49442

Landsell Cryogenics
5303 46th Avenue
Hyattsville, MD 20781



Pumping and distillation assembly. (Dual system. Single distillation setup may be used.)

a, laboratory jack; b, McLeod gage; c, vacuum pump; e, 3-way stopcock; f, pinch clamp; g, vapor trap; h, liquid nitrogen; i, Dewar flask; j, heating tape; k, thermometer (-20° to 150°); l, 500 mL boiling flask with thermometer well; m, power stat.

(a) Boiling flask, 500 ml with thermometer well and 24/40 standard taper neck. Arthur H. Thomas Co. catalog number 4871-G-31 or equivalent.

(b) Thermometer, -20°C to $+150^{\circ}\text{C}$, 75 mm immersion, Fisher Scientific Co. catalog number 14-985-56 or equivalent.

(c) Vapor traps, Arthur H. Thomas Co. catalog number 9466-R75, or equivalent. These traps are fitted with "O" ring ball and socket joints, size 18/9, from Kontes Glass Co., catalog number K-167300 and K-167500, or equivalent.

(d) Adapter, Kontes Glass Co. catalog number K-183000 (or equivalent), fitted with standard taper 24/40 joint to flask and 18/9 "O" ring ball and socket joint to vapor trap.

(e) Vacuum pump, Welch Duo Seal, catalog number 1400B, 25 liters/minute free air displacement. Fisher Scientific Co. catalog number 1-096 or equivalent.

(f) Stopcock, 3-way, with Teflon plug size 2. Kontes Glass Co. catalog number K-82300 or equivalent.

(g) Vacuum tubing, 5/16 in. bore. Fisher Scientific Co. catalog number 14-175D or equivalent.

(h) Vacuum controller, cartesian type. Cole Parmer Instrument Co. catalog number 909 or equivalent.

(i) McLeod gauge, shielded, Roger Gilmont Instruments catalog number G1400B or equivalent.

(j) Heating mantle, 500 ml size, Kontes Glass Co. catalog number K-726000 or equivalent.

(k) Heating tape, double element, type 12, $\frac{1}{2}$ inch wide \times 2 feet long, with finished end and fitted with plug and cord. Kontes Glass Co. catalog number K-729500 or equivalent.

(l) Dewar flask, 4 liter capacity, 17 cm O.D., 15 cm I.D., and 33 cm high. Fisher Scientific Co. catalog number 10-195D or equivalent.

(m) Laboratory jack, Lab Lift, Fisher Scientific Co. catalog number 14-673-10 or equivalent.

(n) Autotransformer, Powerstat type 3PN117B, Arthur H. Thomas Co. catalog number 9461-F-15 or equivalent.

(o) Separatory funnel, 125 ml capacity. Kimble Products catalog number 29048-F or equivalent.

(p) Funnel, Buchner, 60 ml capacity, coarse porosity fritted disc. Kontes Glass Co. catalog number K-955000 or equivalent.

(q) Evaporative concentrator, Kuderna-Danish, 250 ml capacity, 24/40 standard taper column connection, 19/22 lower standard taper joint. Kontes Glass Co. catalog number 570001 or equivalent.

(r) Concentrator tube size 425, 19/22 standard taper joint, 4 ml capacity, Kontes Glass Co. catalog number K-570050 (or equivalent) with 19/22 standard taper stopper. Kontes Glass Co catalog number K-850500 or equivalent.

(s) Distillation column—Snyder, with 24/40 standard taper joint, 3 sections, size 121 Kontes Glass Co. catalog number K-503000 or equivalent.

(t) Water bath, Freas Bath, GCA-Precision Scientific catalog number 66566 or equivalent.

(u) N-Evap concentration equipment with water bath. Supplied with water bath #A-11151, Teflon covered needles #10603, and thermometer #1111, Organomation Associates or equivalent.

(v) Miscellaneous laboratory items including but not restricted to: Teflon sleeve for standard taper joints, pinch clamp No. 18 Arthur H. Thomas Co. catalog number 2841-A-19 or equivalent, and extension type pinch clamp No. 18, Arthur H. Thomas Co. catalog number 2841-E-77 or equivalent, hypodermic syringes, Pasteur pipettes, etc.

(w) Gas Chromatograph, Shimadzu GC-4C PR 6 with column oven autocooling, temperature programmer, removable analytical cartridge, and including: injection port, temperature controller for injection port, and automatic door opener. Glass lined heated transfer line from gas chromatograph to TEA. Dual column system with differential flow controllers, rotameters, and pressure gauges. Glass columns 2.7 mx 3 mm I.D. (5 mm O.D.) or equivalent. Recorder, R 11 m, single pen, 1 mv full scale. The gas chromatograph and its accessories are available from Shimadzu Scientific Instruments. Equivalent gas liquid chromatographic equipment can be substituted for the Shimadzu equipment.

(x) Thermal Energy Analyzer (TEA) Model 502 with one direct sample injection mode and two gas chromatograph interface modes. This is the only sole source item in this list and is essential to this particular analysis. The TEA was obtained from Thermo Electron Corporation.

5.020C. Reagents

(a) Paraffin base mineral oil, white, heavy Saybolt Viscosity 335/350 Laboratory grade (Fisher Scientific catalog number 0-120) or equivalent.

(b) Liquid nitrogen.

(c) Methylene chloride, Burdick & Jackson or equivalent.

(d) Anhydrous sodium sulfate analytical reagent grade, Mallinckrodt catalog number 8024 or equivalent, available from Arthur H. Thomas Co.

(e) Carborundum, #12, granules. Arthur H. Thomas Co., catalog number 1590-33 or equivalent.

(f) Mercury, reagent grade Fisher Scientific Co. catalog number M-141 or equivalent.

(g) Iso-octane, purified grade.

(h) Sodium Hydroxide, Pellets certified ACS Fisher Scientific Co. catalog number S-318 or equivalent.

(i) Column packing material, for GLC: 10% Carbowax 20 M and 5% KOH on Analab AB 100/120 support or equivalent.

(j) N-nitrosamine standards.

(k) Stock mixed standard solution of N-nitrosamine compounds, 5 $\mu\text{g}/\text{ml}$ of each compound of interest, in iso-octane to be obtained directly from NCI, after clearance through FSIS.

(l) Stock standard solution of N-dipropylnitrosamine, 5 $\mu\text{g}/\text{ml}$, in iso-octane. Obtained directly after clearance through FSIS, by writing to:

Mr. Steve Graves
NCI Chemical Carcinogen Repository
Midwest Research Institute
425 Volker Boulevard
Kansas City, Missouri 64110

(m) Working level mixed standard solution. Dilute solution (l) 1 to 20 with methylene chloride.

(n) Working solution of N-dipropylnitrosamine, used as internal standard. Dilute solution (k) 1 to 10 with methylene chloride.

5.020D. Procedure

Notes:

The following procedures contain a number of hazardous steps. All basic safety precautions should always be used. A precautionary technique for preventing injury from imploding Dewar flasks is to wrap the outside of the flask with monofilament (glass fiber) tape.

Extreme care should be exercised in handling nitrosamines or solutions of nitrosamines. These compounds are potent carcinogens. Wearing of rubber gloves is not, of itself, protection from nitrosamines. Every person handling nitrosamines, in any form, should read carefully references 8, 9, and 10.

N-nitrosamines are degraded by ultraviolet (UV) light and exposure of extracts or standard solutions to sources such as sunlight and fluorescent lighting should be avoided.

All reagents used in these analyses must be checked prior to use to assure that no extraneous compounds are present.

All glassware should be thoroughly cleaned, then rinsed with methylene chloride prior to use.

5.020D.1 Distillation

Note: Two mineral oil distillation assemblies are connected to one vacuum pump (e). See Figure 1. A higher vacuum (i.e. lower pressure than 2 torr) is maintained throughout steps 5020D(c)-(d).

(a) Place a 25 g sample in the boiling flask (5020.A(a)). Add 0.5 ml of solution of dipropyl nitrosamine in methylene chloride (0.5 µg/ml); add 2.0 ml of a 0.2 N solution of NaOH; cover the sample with 25.0 ml of mineral oil.

(b) Wet inside of trap with about 2 ml of distilled water. Connect trap to vacuum line and raise Dewar flask so that trap is at least 3/4 immersed in liquid nitrogen. Allow trap to come to equilibrium in the liquid nitrogen. Attach boiling flask and adapter to the trap. Wrap adapter with heating tape. Position heating mantle on the flask. Apply vacuum and check for leaks as system comes to operating pressure. Maintain vacuum on sample 10 min. before applying heat.

Note: Vacuum leak difficulties are sometimes encountered when Teflon sleeves are used with standard taper joints. If necessary vacuum grease can be used instead of Teflon sleeves.

(c) Raise the temperature in the boiling flask, as indicated by thermometer in oil filled well, from ambient to 120°C over a period of 55-60 minutes. Heating tape temperature 170°-175°C.

(d) At the end of the heating period, lower the heating mantle away from the flask. Allow the flask 15 minutes to cool while the adapter remains heated (170°-175°C) and vacuum is maintained.

(e) Carefully release the vacuum and lower the Dewar flask away from trap. Disconnect flask and invert adapter. Allow the trap to warm until the contents are liquid.

5.020D2. Transfer, extraction and drying

NOTE: Subsequent sections describe washing or rinsing of trap, and extractions in separatory funnels. In the washing or rinsing of trap, a one-minute agitation of the contents is required to assure the best possible washing of all interior surfaces. All extractions in separatory funnels require one minute of vigorous shaking, followed by ample time for complete separation of the layers before removing the lower layer.

(a) Rinse the adapter with 10 ml of methylene chloride collecting the rinsings in the trap.

(b) Transfer distillate and methylene chloride washing to a 125 ml separatory funnel.

(c) Rinse the trap with 15 ml of methylene chloride: 5 ml through the stem, 10 ml through the body of tube.

- (d) Transfer the methylene chloride to the separatory funnel and extract the aqueous distillate by shaking for 1 min.
- (e) Allow phases to separate. Transfer lower methylene chloride layer to a second 125 ml separatory funnel.
- (f) Perform 5.020D 2(c) to (e) sequence a total of three times.
- (g) Set up the Buchner funnel with 30 g of anhydrous sodium sulfate. Wash the anhydrous sodium sulfate with 25 ml of methylene chloride.
- (h) Set up a Kuderna-Danish flask fitted with the concentrator tube (5.020A(r)).
- (i) Suspend the Buchner funnel containing the washed sodium sulfate over the Kuderna-Danish flask so that all liquid filtered through will be collected in the flask.
- (j) Dry pooled methylene chloride in second separatory funnel by passing it through washed sodium sulfate into Kuderna-Danish flask.
- (k) Rinse second separatory funnel with 25 ml of methylene chloride. Pass it through the sodium sulfate into Kuderna-Danish flask.

5.020D.3 Concentration of the extract

NOTE: Several precautions are necessary in this section. Perhaps the most important one is:

FROM THIS POINT ON, DO NOT ALLOW WATER TO GET INTO THE EXTRACT.

Anytime the apparatus is subjected to condensation or is exposed to steam or other water vapor, caution is necessary to avoid water on ground joints or other entry into the extract.

- (a) Add one grain of carborundum to the Kuderna-Danish concentrator and fit the flask with a Snyder column.
- (b) Suspend the Kuderna-Danish apparatus in a 60°C water bath. Adjust the depth of immersion of the apparatus, so that the volume of the extract will be reduced to 4.0 ml in one half to one hour.

NOTE: Water baths are available in many varieties. The one listed is a specific kind. It contains heated water under a relatively tight top. The top is fitted with concentric rings, so that a flask may simply be placed on a hole of appropriate size. Other baths may be substituted if adequate results are obtained.

- (c) Remove the apparatus from the bath, wipe dry, and allow to cool to ambient temperature.
- (d) Transfer the concentrator tube from the Kuderna-Danish apparatus to the N-Evap system with the water bath set at 35°C-40°C. Further reduce the volume of the extract to 1.0 ml over a period of approximately 30 minutes.

5.020E Gas chromatographic separation of the nitrosamines and detection by the TEA.

NOTE: Gas chromatographic conditions are extremely important. Resolution between N-nitrosopiperidine and N-nitrosophyrrolidine must be at least 0.8. Conditions must be at least this good before using the chromatograph, must be checked periodically, and when column degradation or other conditions cause to GLC to fall below these specifications, a new column or returning must be provided. Operating conditions and an equation for calculating resolution are presented in 5.020E.

(a) Inject 6.0 microliters of the working mixed standard solution (0.25 $\mu\text{g}/\text{ml}$ of each nitrosamine of interest). Obtain from this injection, the retention time and response of each compound of interest.

NOTE: As of this date, the list of nitrosamines of interest includes dimethylnitrosamine, diethylnitrosamine, di-propylnitrosamine, dibutylnitrosamine, N-nitrosopiperidine, N-nitrosopyrrolidine, and N-nitrosomorpholine. Di-propylnitrosamine is also used as an internal standard.

(b) Inject 6.0 microliters of the concentrated sample extract. Obtain from this injection, the retention time and response of each compound of interest that is present.

(c) Calculate the amount of each compound of interest present in the sample extract, and relate the amount to its level in the original sample, i.e. convert nanograms in the injection to parts per billion (ppb) in the original sample.

5.020F References

1. Havery, D. C., Fazio, T., and Howard, J. W. Survey of Cured Meat Products for Volatile N-Nitrosamines: Comparison of Two Analytical Methods. Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, Publication date 1978.

2. Gough, Terry A., Webb, Kenneth S., Pringer, Martin A., and Wood, Beryl J.A. Comparison of Various Mass Spectrometric and a Chemiluminescent Method for the Estimation of Volatile Nitrosamines. J. Agric. Food Chem., Vol. 25, No. 3, 1977, 663-667.

3. Fine, D.H., Rounbehler, D.P., and Oettinger, P.C. A Rapid Method for the Determination of Sub-Part-per-Billion Amounts of N-Nitroso Compounds in Foodstuffs Analytical Chimica Acta, 78 (1975) 383-389.

4. Private communications to Mr. E. L. Greenfield from Dr. W. Fiddler, USDA, SEA, ERRD, Philadelphia, PA. Nov. 1978.

5. Private communications to Mr. E.L. Greenfield from T. Fazio, D. Havery, Dept. of HEW, FDA, Wash., DC Dec. 1978.

6. National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. Department of Health, Education, and Welfare Publication (NIH) 75-900 (1975).

7. Steere, N. V. (1974). Safety in the Chemical Laboratory CXVI—Occupational Safety and Health Standards Adopted for Fourteen Carcinogens. J. Chem. Educ. 51 (6), A322-A325.

8. Gough, T. A., Webb, K. S., and McPhail, M. F. Diffusion of Nitrosamines through Protective Gloves Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, Publication date 1978.

9. Walker, E. A., Castegnaro, M., Garren, L., and Pignatelli, B. Limitations to the Protective Effect of Rubber Gloves for Handling Nitrosamines. Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, Publication date 1978.

10. Sansone, E. B. and Tewari, Y. B. The Permeability of Laboratory Gloves to Selected Nitrosamines. Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, Publication date 1978.

5.020G. Gas Liquid Chromatographic (GLC) Parameters

Typical GLC conditions, in use in FSIS laboratories

Injector	185°C
Column	165°C
Interface line from column to TEA	230°C
Recorder	1 mv
Chart speed	5mm/min
Carrier gas	N ₂ 40 cc/min
GLC column:	

Column 2.7 m × 3.0 mm I.D. packed with Analab AB 100/120 mesh coated 10% Carbowax 20M and 5% KOH or equivalent.

Chromatographic column should not be used when:

The resolution between N-nitrosopiperidine and N-nitrosopyrrolidine, calculated as follows:

$$R = \frac{T_2 - T_1}{1/2(W_1 + W_2)}$$

is less than 0.8.

Where R = Resolution

T₁ = Retention time (mm) N-nitrosopiperidine

T₂ = Retention time (mm) N-nitrosopyrrolidine

W₁ = Peak width at base (mm) N-nitrosopiperidine

W₂ = Peak width at base (mm) N-nitrosopyrrolidine

5.021 Determination of Nitrosamines by the Low Temperature Vacuum Distillation Procedure

5.021A. Theory

This procedure uses a low temperature distillation technique developed by N.P. Sen (See Reference 2) to recover the volatile N-nitrosamines from various meat products. Detection and quantitation are accomplished by the use of GC-TEA (See "Chemistry Laboratory Guidebook" Sec 5.020). An additional column cleanup of the extract is adequate for mass spectral confirmation.

5.021B. Apparatus

(Note: Glassware catalog numbers refer to Kontes catalog TG-50 unless otherwise noted. Equivalent glassware may be substituted.)

(a) *Distillation Apparatus* — See Figure 1

1. All glass, flash evaporator, vertical, with adjustable heating bath (Buchler (PTFE-1 GN, or equivalent).
2. Two liter round bottom flask with 24/40 joint (K-601001).
3. 250 ml round flask with 24/40 joint (K-601001).
4. Needle valve to adjust air bleed (Pyrex rotoflo (Corning 7740), or equivalent).
5. Ice bath with circulating water pump (Little Giant 501003, or equivalent).
6. Vacuum pump, minimum 25 L/min capacity (GCA/Precision Scientific, Vac Torr D-25, or equivalent).
7. Vacuum control cartesian (Gilmont C-2100, or equivalent).
8. Vacuum gauge capable of monitoring pressure of 20 Torr. A manometer (Dwyer Series 1222 model M-1000, or equivalent) provides satisfactory service. Requires calibration with barometer or MacLeod gauge immediately before use.

(b) *Extraction and Cleanup Apparatus*

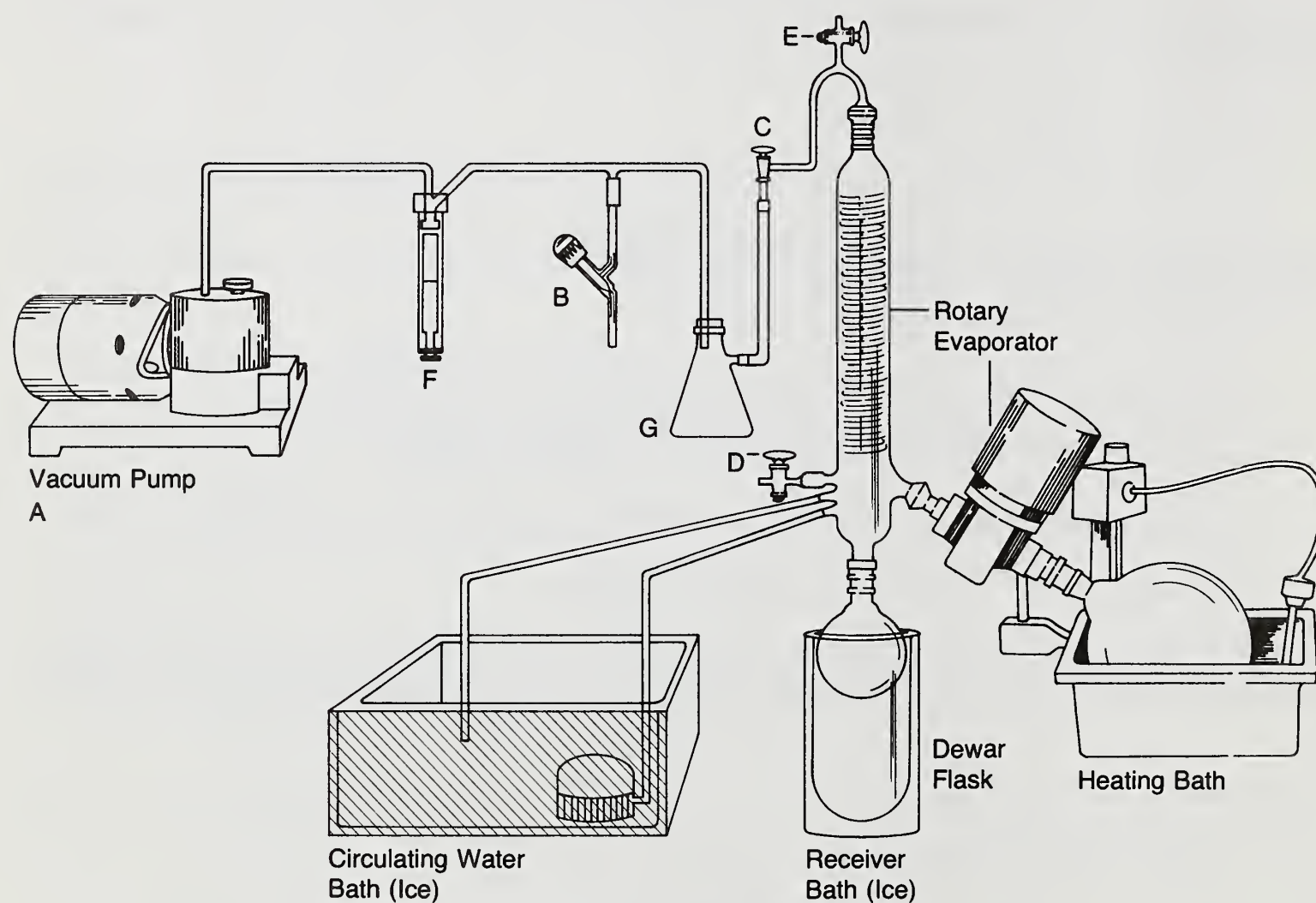
1. Syringe, all glass, 50 ml capacity.
2. 250 ml graduated mixing cylinder (K-482000).
3. 500 ml separatory funnel with Teflon stopcock (K-636030).
4. Glass filtering funnel, 150 ml, with 6 mm coarse porosity fritted disc (K-955000).

(c) *Evaporative Concentrators*

1. 250 ml Kuderna Danish evaporative concentrator (K-570001).
2. 500 ml Kuderna Danish evaporative concentrator (K-570001).
3. 4 ml graduated concentrator tube (K-570050-425).
4. Snyder column, large 3 section with 24/40 joint (K-503000-0121).
5. Snyder column, micro 3 section with 14/20 joint (K-569251).

Figure 1

Distillation Apparatus



- A—Vacuum Pump
- B—Variable Air Bleed
- C—Isolation Valve
- D—Vacuum Take-Off (Calibration)
- E—Vent
- F—Vacuum Regulator (Cartesian)
- G—Trap

(d) *Chromatographic Apparatus*

1. Chromatographic column, 10.5 mm ID. × 250 mm length with 200 ml reservoir (K-420280-213).
2. Chromatographic column 22 mm ID. × 300 mm length (K-420540-233).
3. Pasteur pipet, disposable, 9 inch (K-88350).

(e) Water bath, circulating, with temperature controller. (Precision model 260, or equivalent).

(f) GC/TEA Shimadzu 4 CM gas chromatograph, or equivalent, interfaced to Thermo Electron 502 thermal energy analyzer, GC Conditions: 2.7 m × 3 mm I.D. glass column packed with 10 percent Carbowax 20 M + 5% KOH on 100/120 chromosorb P AW; injection port 200°C; interface 280°C isothermal; nitrogen carrier gas 25-30 ml/min TEA conditions: furnace 470°C; liquid nitrogen trap; initial and working vacuum settings and oxygen flow must be determined for particular instrument being used.

Recorder response should be >10% for 1.5 ng N-nitrosopyrrolidine. Resolution (R) between N-nitrosopiperidine and N-nitrosopyrrolidine should not be <0.8 when calculated as follows:

$$R = \frac{T_2 - T_1}{\frac{1}{2}(W_1 + W_2)}$$

where T_1 and T_2 = retention times (mm) of N-nitrosopiperidine and N-nitrosopyrrolidine; W_1 and W_2 = peak width as base (mm) of N-nitrosopiperidine and N-nitrosopyrrolidine.

(g) GC/MS Pye Unicam 104 interfaced to AEI MS 30 by all glass jet separator. GC Conditions — 9 ft. × 3 mm I.D. glass column packed with 5% FFAP on 60/80 chromosorb W HP; injection port 160°C, column temperature 145°C isothermal; jet separator 180°C; helium flow 18 ml/min. MS Parameters:

MS 30: Temperature at ion source = 200°C
Resolution of 10% valley definition = 3300
Temperature at jet separator = 180°C

(h) GC/MS—Hewlett-Packard 5992 with capillary interface and splitless injector. GC conditions: Column = 0.2mm × 25m capillary coated with carbowax 20m; head pressure = 20 psig; injection port = 145°C; vent delay = 40 seconds; temperature program = 5 min. held at 90°C, then programed to 170°C at 5°C/min; solvent vent 2 min; Begin data acquisition at 2.5-5.0 minutes, depending on size of solvent front.

5.021C. Reagents

(a) N-nitrosamine standards to be obtained directly from IITRI, after clearance through FSIS, from: Program Manager, Chemical Repository, Illinois Institute of Technology Research Institute, 10 W 35th Street, Chicago, Illinois 60616. Mixed standards consist of approximately 5 µg/ml each of N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosodibutylamine (NDPA), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR) and N-nitrosomorpholine (NMOR), in iso-octane. N-nitrosodipropylamine standard consists of approximately 5 µg/ml NDPA in iso-octane. N-nitrosodipropylamine standard consists of approximately 5 µg/ml NDPA in iso-octane. Prepare working standards by making appropriate dilutions with methanol or dichloromethane.

Solvents note: All solvents listed are glass distilled (Burdick and Jackson Laboratories, Muskegon, MI).

1. Dichloromethane (DCM). Concentrate 200 ml of each lot to 0.5 ml and check for interfering peaks. Extract with equal volume 6N HCl prior to use. Discard the HCl layer.

2. Pentane. Concentrate 100 ml of each lot to 1 ml and check for interfering peaks.

3. Methanol, anhydrous.

4. Hexane

5. Acetone

(c) Water, glass distilled. Do not pass through ion exchange resins.

(d) Potassium hydroxide, reagent grade. Prepare 3N and 1N solutions.

(e) Hydrochloric acid, concentrated, reagent grade. Prepare 6N solution.

(f) Phosphoric acid, reagent grade. Prepare 6N solution.

(g) Alumina, basic activity I (Fisher Scientific Co.) Heat overnight at 300°C. Deactivate 6 percent (w/w) with distilled water. Store in stoppered container in desiccator. Alumina is suitable for chromatography if cleanup procedure, (See Alumina Column Chromatography), using 1.0 ml mixed nitrosamine std. ($\sim .25 \mu\text{g/ml}$ in DCM) yields at least 85 percent recovery of NDMA, NPIP, NPYR, and NMOR, with no traces of these compounds in the first eluate, and reagent blank shows no interfering peaks.

(h) Celite 545, not acid washed (Fisher Scientific Co.). Activate overnight at 700°C. Store in stoppered container in desiccator. Celite is suitable for chromatography if cleanup procedure, (See Acid Celite Chromatography), using 1.0 ml mixed nitrosamine standard ($\sim .25 \mu\text{g/ml}$ in DCM) yields at least 85 percent recovery for NDMA, NPYR, and NMOR, and reagent blank shows no interfering peaks.

(i) Silicon carbide granules, 12 mesh (Hengar Granules), or equivalent.

(j) Glass wool, fine, for column chromatography.

(k) Ice cubes, crushed ice, or dry ice for cooling bath.

(l) Sodium sulfate, anhydrous, granular, reagent grade.

5.021D. Determination

(a) Adjusting Distillation Parameters

Refer to Figure 1. Assemble distillation apparatus as shown. Attach vacuum gauge or manometer (note: manometer must be calibrated for zero pressure immediately before use to minimize possible error due to changes in barometric pressure) at (D) to monitor pressure within rotary evaporator. Apply vacuum to system and adjust air bleed (B) to maintain an internal pressure at 19-20 torr. Vacuum should remain quite stable once set. Recheck system for proper pressure at least weekly. Fill water bath to normal operating level and adjust regulator to maintain a bath temperature of 45-46°C. (Bath temperature may require readjustment during distillation.)

(b) Distillation

Weigh 25 g sample into a two liter round bottom flask. Add 200 ml 3N KOH and 1.0 ml NDPA ($0.25 \mu\text{g/ml}$ in methanol). Attach distilling flask to evaporator and 250 ml round bottom receiver to condenser. Immerse receiver in an ice bath (0-3°C) and begin circulation of ice water through condenser. Immerse distilling flask in heating bath 45-46°C, (Critical Step - Temperature must be maintained at no lower than 45°C), apply vacuum and turn on rotary evaporator.

Adjust air bleed (B), if necessary, to insure that vapors condense no further than one third the way up the condenser. If excessive foaming occurs, reduce vacuum with air bleed, as necessary, to prevent mechanical transfer of distillate, until foaming condition diminishes. As distillation proceeds, increase vacuum until full vacuum (20 torr) is reached. Full vacuum should be reached within 10 minutes of onset of distillation. Continue distillation until drip rate from condenser averages less than one drop per minute. Total distillation time should be about one hour. When distillation is complete, turn off rotary evaporator. Disconnect vacuum, using isolation valve (C), release vacuum, using vent (E), and remove receiver. Transfer contents of receiver to a 250 ml mixing cylinder. Reattach receiver to condenser, and using a large syringe, thoroughly wash down the walls and coils of the condenser with about 160 ml DCM, taking care to wash out all remaining aqueous distillate. Remove receiver and save for following step.

(c) Extraction and Cleanup

Acidify aqueous distillate with 4 ml concentrated HCl and mix by shaking. Note volume, then transfer to a 500 ml separatory funnel. Fill mixing cylinder with DCM rinse from condenser. Add additional DCM, if necessary, until volume is equal to that of acidified distillate. Mix by shaking and add to aqueous distillate in separatory funnel. Extract distillate by shaking vigorously for 1 minute. Allow to stand until layers separate, then drain DCM layer into a second 500 ml separatory funnel. Extract distillate with a second equal volume of DCM as before, and pool extracts. Discard distillate. Wash combined extracts with 50 ml 6N HCl, shaking vigorously for 1 minute. After phases separate, drain lower layer into a third 500 ml separatory funnel. Backwash acid twice with 50 ml portions DCM, shaking 1 minute each time. Add first backwash to extract in third funnel. Save second backwash to extract KOH solution in following step.

Wash DCM in third funnel with 50 ml 1N KOH, shaking 1 minute. Allow phases to separate, then drain DCM phase through a 60 mm glass fritted funnel containing 30 g sodium sulfate (prewashed with 30 ml DCM) into a 500 ml Kuderna Danish concentrator fitted with a 4 ml graduated concentrator tube. This is necessary to remove traces of water from the extract. Drain second acid backwash into third funnel now containing 50 ml KOH. Shake 1 minute and allow phases to separate. Drain DCM layer through sodium sulfate into concentrator.

Add 1 ml hexane and a silicon carbide granule, attach Snyder Column, and concentrate on a circulating water bath at 60-65°C, to 4-5 ml at a rate not to exceed 3 ml per minute. Allow vapor to condense completely. Remove concentrator tube, attach micro Snyder Column, and add a second silicon carbide granule. Immerse tip of tube in 60°C-65°C bath and concentrate to about 0.8-0.9 ml (Critical Step—Do Not Evaporate with Nitrogen). Remove from bath and wash down column with a few drops DCM. Cool, and adjust volume to 1.0 ml with DCM. Stopper tube.

(d) Quantitation

Inject a suitable aliquot on GC-TEA and quantitate all nitrosamines. Use standards at $\sim 0.25 \mu\text{g/ml}$ in DCM for quantitation. NDPA recovery must be at least 80 percent, or distillation should be repeated. If samples require dilution for quantitation, dilute with *n*-pentane, not DCM, or additional cleanup for mass spectral confirmation will be compromised. If samples must be stored overnight prior to further cleanup, add sufficient *n*-pentane to bring volume to 4-5 ml. Samples containing any nitrosamine in concentrations equal to or exceeding 9.5 ppb are violative, provided that the presence of the nitrosamine can be positively confirmed by mass spectrometry. When a sample requires confirmation, the tissue blank and recovery which have been analyzed concurrently must also be analyzed as confirmation samples.

(e) Mass Spectral Confirmation—Additional Required Cleanup

The nature and amount of interfering substances remaining in the concentrate after initial distillation and cleanup varies markedly from sample to sample. Most bacon samples will require further cleanup prior to successful confirmation by mass spectrometry. Cleanup procedures using alumina and acid-celite column chromatography have been investigated. Acid celite usually provides better overall cleanup for samples containing NPYR and NMOR, while alumina is more effective when NDEA, NDPA, NDBA, or NPIP are present. The column of choice for samples containing NDMA is best determined experimentally by the analyst.

(f) Alumina Column Chromatography

Add a small glass wool plug to a 10.5 mm \times 250 mm chromatographic column. Rinse column with 5 ml DMC, then 5 ml *n*-pentane, allowing rinses to drain. Close stopcock and add 15 ml pentane to column. Add 5 g alumina (use of a

long stem funnel is helpful) and allow to settle. Wash down any alumina that may adhere to column walls with a small amount of n-pentane. Add a layer of sodium sulfate about 1 cm thick to top of column, then drain n-pentane to a level 1 mm above top of sodium sulfate. Add 3 ml n-pentane to column.

Using a Pasteur pipet, transfer sample (mixed with 3 ml pentane) to column. Rinse concentrator tube twice with 2 ml n-pentane, adding rinsings to column. If sample contains NDEA, NDPA, or NDBA, place a 250 ml Kuderna Danish apparatus with 4 ml concentrator tube under column. Drain sample onto column at 60-80 drops per minute until level is 1 mm above top of sodium sulfate, wash down walls of column with 2-3 n-pentane and drain to same level.

Add 50 ml 20 percent DCM in n-pentane to column and elute at 60-80 drops per minute until level reaches top of column. Momentarily stop flow and replace concentrator, if used, with another Kuderna-Danish apparatus. Add 60 ml DCM to column and drain into concentrator at 60-80 drops per minute. If initial fraction was collected, (for NDEA, NDPA, NDBA), add 25 ml DCM to that fraction. Add 1 ml hexane and a silicon carbide granule to all fractions collected and concentrate to 4-5 ml on a 55-60°C circulating water bath at a maximum rate of 3 ml/minute. Concentrate to approximately 0.5-1.0 using a micro Snyder column as previously described.

(g) Acid Celite Chromatography

Weight 5.0 celite into a 150 ml beaker. Add 7.5 ml 6N H_3PO_4 in approximately 2.5 ml portions, stirring vigorously with a glass rod to assure a uniform mixture that is fluffy in consistency. Pack mixture into a 22 mm I.D. \times 300 mm chromatographic column containing a small glass wool plug. Compress celite to 4.5 cm, placing a small glass wool plug on top of absorbent. Add 10 ml n-pentane to column, and drain until liquid layer is 1 mm above top of celite layer. Using a Pasteur pipet, quantitatively transfer sample onto column using sufficient pentane so that the volume of sample plus washes is approximately 10-12 ml. Drain sample onto column at 60-80 drops per minute, stopping when liquid level is 1 mm above celite. Wash column walls with 3-4 ml pentane, draining to previous level. Add 70 ml 2 percent DCM in pentane to column and drain at 60-80 drops per minute. Pass 60 ml DCM through column, collecting eluate in a 250 ml Kuderna-Danish apparatus. Add 1 ml hexane and concentrate as described under alumina column chromatography.

(h) Preparing samples for Mass Spectrometry

Transfer concentrate from (f) or (g) to a 1 ml reaction vial or centrifuge tube graduated in increments of at least 0.1 ml. Adjust the volume of the sample so that its nitrosamine concentration matches that of the standard to be used for the confirmation. (For example, if the standard injected has a nitrosamine concentration of 5 $\mu\text{g/ml}$, a sample quantitated at 10 ppb will have to be reduced in volume to 50 μl). Concentrate the sample slowly, using a gentle stream of dry nitrogen.

(i) Mass Spectrometry

Successful confirmation by mass spectrometry requires that both the GC retention and the monitored ion fragments of the sample match those of an injected standard. The possibilities of false positives should be compensated for by injections of a tissue blank and a recovery, respectively, in the same run. Both a magnetic sector instrument operating at medium resolution and a quadrupole have been used for confirmation. Parameters for both are listed below.

1. Confirmation parameters—AEI MS 30

a. Calibration of the mass spectrometer

The mass spectrometer should be calibrated with perfluorokerosene (PFK) or other suitable calibration compounds for the mass range of interest with the 10% valley definition resolution better than 3000, to separate 29.9980 to 30.0343 and 28.0188 to 28.0061 (H_2^+ , ionized from the residual air in vacuum system).

b. Confirmation of nitrosamines in study sample

The gas chromatographic retention time of a purported nitrosamine in a study sample should be within $\pm 2\%$ of the nitrosamine in the standard sample or fortified blank.

The presence of the molecular ion (M^+) (or quasi-molecular ion $(M+H)^+$ in case of using chemical ionization mode) and the most significant characteristic fragment ions for nitrosamines at m/z 29.9980 (NO^+) and at least two other fragment ions must be detected with the signal to noise ratio larger than two. These additional two-fragment ions are usually of the following ions: m/z 28.0188 (CH_2N^+), 29.0266 (CH_3N^+), 30.0343 (CH_4N^+), 41.0266 ($C_2H_3N^+$), 42.0344 ($C_2H_4N^+$), 43.0422 ($C_2H_5N^+$), 44.0500 ($C_2H_6N^+$) and the $(M-NO)^+$, $(M-HNO)^+$, and $(M-H_2NO)^+$ ions.

2. Confirmation parameters—HP 5992 (not applicable to DMNA, DENA)

a. Calibration of mass spectrometer

Successful autotune calibration must be achieved prior to attempt confirmation. Operate at highest practical resolution.

b. The CC retention time of the purported nitrosamine in the sample should be within $\pm 3\%$ of the standard or recovery. A minimum of 4 characteristic ions, including the molecular ion, must be detected. Ions monitored for each compound are: DBNA-158, 116, 74, 30; NPIP-114, 74, 42, 30; NPYR-100, 68, 42, 30; NMOR-116, 86, 42, 30. Relative intensities of the ions monitored must match those of the standard or recovery. The match is acceptable for confirmation if the ratios of the intensities of the three fragment ions relative to the molecular ion are within $\pm 20\%$ of those of the standard or recovery.

References

1. Sen, N.P., Private Communication Canadian Dept. of Health and Welfare, Food Research Division, Ottawa, Ont.
2. Sen, N.P., et al., J. Agric. Food Chem. Vol. 27, No. 6, 1979, pg. 1354-1357.

5.022 Quantitation of Chloramphenicol in Bovine, Swine, and Poultry Muscle Tissue by Electron Capture GLC.

5.022A. Theory

This is a method developed by Jacobson, Allen and Wiseman at FDA, Bureau of Veterinary Medicine, and modified by Simpson, Ali and Rivera-Mendez, USDA-FSIS-Science-Eastern Laboratory. After the addition of an internal standard, the monochloro derivative of chloramphenicol, the tissue is extracted with ethyl acetate, the ethyl acetate evaporated to dryness, and the residue redissolved in 4 percent aqueous sodium chloride. The aqueous phase is partitioned three times against hexane and the hexane discarded. Chloramphenicol is extracted from the aqueous phase with ethyl acetate. The ethyl acetate is taken to dryness. Quantitation is by electron capture gas liquid chromatography following formation of the trimethylsilyl derivative. Samples found in violation are confirmed by mass spectrometric analysis.

5.022B. Apparatus

- (a) 100 ml glass centrifuge tube (Kontes K411050, or equivalent).
- (b) Culture tube, screwcap, with Teflon liner, 25 × 150 mm (Corning 9826, or equivalent).
- (c) 15 ml glass stoppered glass centrifuge tube (VWR 20922-029, or equivalent).
- (d) Centrifuge-Damon/IEC model HN-S equipped with eight place fixed angle rotor, or equivalent.
- (e) Mechanical Shaker — Eberbach flat bed (VWR 57007-000, or equivalent).
- (f) N-Evap, Model 11 (Organomation Assoc. Inc., or equivalent).
- (g) Polytron (Brinkman Industries, equipped with small blending head, or equivalent).
- (h) Gas Chromatograph (Hewlett Packard model 5840 equipped with electron capture detector), or equivalent, equipped with automatic sampler.
- (i) Column: 6 ft. by 2 mm id, packed with 3% OV-101 on 100/120 mesh Gas Chrom Q. Minimum acceptable theoretical plates for column is $N = 3000$. Injection of 0.125 ng of derivatized chloramphenicol standard under the conditions described in 5.022G should produce a peak height of approximately 40 mm, with a signal to noise ratio of 5:1, or more.

5.022C. Reagents

- (a) Ethyl Acetate — distilled in glass (Burdick & Jackson, or equivalent).
- (b) Methanol — distilled in glass (Burdick & Jackson, or equivalent).
- (c) Hexane — distilled in glass (Burdick & Jackson, or equivalent).
- (d) Sylon HTP — Supelco, Inc. (#3-3-43, or equivalent).

Hexamethydisilazane (HMDS)	3 Parts
Chlorotrimethylsilane (TMCS)	1 Part
Pyridine	9 Parts

- (e) Sodium Chloride — ACS grade, 4 percent aqueous solution.
- (f) Chloramphenicol — analytical standard — Sigma Chemical Company, St. Louis, MO. 63178.

(g) Monochloro derivative of Chloramphenicol — USDA-FSIS-Science-Eastern Laboratory, P.O. Box 6085, Athens, GA, 30604.

(h) Cyclohexane — distilled in glass — (Burdick & Jackson, or equivalent).

5.022D. Preparation of Standards

(a) Stock Solutions

(1) Into a 100 ml volumetric flask, weigh 50.0 mg of chloramphenicol analytical standard. Dissolve and make to volume with methanol (500 ug/ml).

(2) Into a 100 ml volumetric flask, weigh 50.0 mg of the monochloro derivative of chloramphenicol. Dissolve and make to volume with methanol (500 ug/ml).

(b) Internal Standard (IS) Solutions

(1) Solution A (50 ug IS/ml)

Into a 100 ml volumetric flask, pipet 10 mls of stock solution (a)(2) and bring to volume with methanol. 100 ul of Solution A is added to samples that have violative levels between 0.5-5 ppm.

(2) Solution B (2.5 ug IS/ml)

Into a 200 ml volumetric flask, pipet 10 mls of IS Solution A and bring to volume with methanol. 100 ul of Solution B is added to every sample.

(c) Preparation of Working Standards

(1) Solution C (50 ug Chloramphenicol/ml)

Into a 100 ml volumetric flask, pipet 10 mls of stock solution (a)(1) and bring to volume with methanol.

(2) Solution D (5 ug Chloramphenicol/ml and 2.5 ug IS/ml)

Into a 100 ml volumetric flask, pipet 10 mls of Solution C and 5 mls of IS Solution A, and bring to volume with methanol.

(3) Solution E (2.5 ug Chloramphenicol/ml and 2.5 ug IS/ml)

Into a 100 ml volumetric flask, pipet 50 mls of Solution D and bring to volume with IS Solution B.

(4) Solution F (1.25 ug Chloramphenicol/ml and 2.5 ug IS/ml)

Into a 100 ml volumetric flask, pipet 50 mls of Solution E and bring to volume with IS Solution B.

Note: All standard solutions should be stored in a freezer maintained at 0°C or lower. Allow working standard to equilibrate to room temperature before use.

Caution: Usual glassware washing procedures are not always effective in removing all traces of chloramphenicol from glass surfaces. It is recommended that all glassware be pre-rinsed with methanol before use to avoid contamination problems.

5.022E. Sample Preparation

Muscle tissue should be prepared according to instructions contained in the USDA Laboratory Guidebook, Section 1.002. Tissue should be kept frozen at all times until analyzed.

5.022F. Determination

(a) Weigh 5.0 ± 0.1 g of ground muscle tissue into a clean glass tube and fortify sample with 100 μ l of Solution B (internal standard). Select a blank tissue and fortify three samples of this tissue with 100 μ l aliquots of Solutions D, E, and F.

(b) Add 30 ml ethyl acetate and blend for 1.0 minutes using a Polytron tissue homogenizer.

(c) Centrifuge at 2500 rpm for 5 minutes.

(d) Transfer the clear ethyl acetate layer to clean 25×150 mm screwcap culture tube.

(e) Evaporate ethyl acetate extract to dryness using N-Evap with water bath set for 60-70°C.

(f) Dissolve residue in 2 ml methanol, mix samples. Add 25 ml 4% sodium chloride and 15 ml hexane.

(g) Shake for 5 minutes on mechanical shaker.

(h) Allow phases to separate by standing for 3 minutes.

(i) Remove hexane layer and discard.

(j) Add 15 ml hexane and repeat steps g, h, and i.

(k) Add 15 ml ethyl acetate and shake for 10 minutes.

(l) Allow phases to separate by standing for 5 minutes.

(m) Transfer ethyl acetate to clean 15 ml glass stoppered centrifuge tube and evaporate using N-Evap at 60-70°C.

(n) Wash walls of centrifuge tube with 1 ml methanol and evaporate to dryness.

(o) Wash walls of centrifuge tube with 1 ml hexane and evaporate to dryness.

(p) Add 200 μ l Sylon HTP Stopper tubes. React for 15 minutes at 60-70°C.

(q) Evaporate excess reagents on N-Evap, with care to approximately 0.05 - 0.10 ml. Caution: Excessive drying time at this step may result in loss of analyte.

(r) Reconstitute residue in 2.0 ml 60% cyclohexane and 40% hexane. mix well and transfer to GLC vials (about 1 ml).

5.022G. Gas Liquid Chromatographic Analysis

(a) Operating Conditions for HP 5840

(1) Column Temperature	220°C
(2) Flow Rate	20 ml/min*
(3) Injector Temperature	230°C
(4) Detector Temperature	350°C
(5) Chart Speed	0.5 cm/min
(6) Attenuation	7
(7) Slope Sensitivity	0.75

(b) Inject samples and standards in duplicate using automatic liquid sampler set to inject 5.0 ul.

*Carrier gas is 95:5, Argon:Methane

Note: Approximate retention time 8 ± 2 minutes.

5.022H Calculations

(a) Measure the peak heights of chloramphenicol and the internal standard peaks of each fortified sample and calculate their respective peak height ratios.

(b) Using linear regression, construct a standard curve of chloramphenicol concentration vs. peak height ratio.

the equation: $y = mx + b$

where x = chloramphenicol/IS peak height ratio

y = chloramphenicol concentration (ppb)

m = slope

b = y intercept

The correlation coefficient should be ≥ 0.995

(c) From the measured peak height ratio, using the regression slope and intercept, compute the chloramphenicol concentration (y) for each incurred sample.

(d) From the measured height ratio, using the regression slope and intercept, compute the chloramphenicol concentration (y) for each incurred sample.

5.022I Formulas

(a) Slope

$$m = \frac{n \sum xy - \sum x \sum y}{n \sum x^2 - (\sum x)^2}$$

(b) Intercept

$$b = \frac{\Sigma y}{n} - \frac{m \Sigma x}{n}$$

(c) Regression Coefficient

$$r = \frac{\Sigma xy}{\Sigma x^2 \cdot \Sigma y^2}$$

(d) Standard Error of Estimate

$$s = \sqrt{\frac{1}{n - 2} \left[\Sigma y^2 - \frac{(\Sigma xy)^2}{\Sigma x^2} \right]}$$

5.023 Chloramphenicol Gas Chromatographic/Mass Spectrometry Confirmatory Procedure

5.023A. Theory

This is a method to confirm the presence of chloramphenicol in tissue extracts by quadrupole GC/MS. The method uses a capillary column for separation and negative ion chemical ionization for detection/confirmation. The method is sensitive enough to detect 200 pg of derivatized chloramphenicol. The action level for chloramphenicol is 10ppb (10ng/g). Because the quantitative portion of the method uses a 5 gram sample, sufficient material will be present for confirmation, if chloramphenicol is present.

5.023B. Apparatus

Instrument: Finnigan 4000 GC/MC, or equivalent

GC Conditions and Parameters:

Column: 25 meter OV-101 Fused Silica Capillary

Temperature Parameters:

Injector: 210°C

GC oven: 150°C for 1 minute, then programmed to 255°C at 30°/minute

Separator Oven: 250°C

Transfer Line: 250°C

Column Pressure: 16 psi

Splitless injection - gas flow off prior to injection, and resumes at about 0.8 minutes following injection.

Mass Spectrometer Conditions: Using a source pressure of about 4.6×10^{-5} t. methane enhanced negative ion chemical ionization (NICI) is used to analyze the TMS derivative of chloramphenicol.

The reagent gas is admitted to the source using the makeup gas value to produce a forepressure reading of about 30t.

Other mass spectrometry parameters:

Filament voltage: 40 eV

Emission Current: 0.25 mas

Manifold Temperature: 100°C ($\pm 5^\circ$)

Ionizer Temperature: 270°C

Lenses 3, 4, and 5 and the quad off-set and program voltages adjusted to maximize the ions at m/z 595 and 633 from perfluoroheptabutylamine (FC-43) consistent with minimizing low mass lift-off and preserving and good peak shape.

5.023C. Determination

(a) Because the mass spectrometer is operated in the Multiple Ion Detection Mode, care must be taken to ensure that the monitored ions are "centered" at the monitored mass, and that data is collected over the narrowest "mass window" possible to minimize the possible interference from extraneous materials of the same nominal mass within the sample matrix.

(b) To determine where the monitored masses will be centered, selected masses, as listed, are monitored following injection of 10 or 20 ng of a derivatized chloramphenicol standard.

Masses monitored to determine centering:

465.9	358.1
466.0	358.2
466.1	303.9
466.2	304.0
357.9	304.1
358.0	304.2

(c) The computer should be set so that data is collected with a narrow mass window-zero, if possible. (The use of the "fat peaks" option available on the Hewlett-Packard GC/MS/Computer System is not permissible.) Resolution of the mass spectrometer should be as high as possible, and under no circumstances shall be less than 2 times the monitored mass.

(d) After the mass centers are ascertained, these corrections are applied to all of the monitored masses. The correction obtained for m/z 466 is applied to m/z 468, that obtained for m/z 358 is applied to m/z 360, and that obtained for m/z 304 is applied to m/z 322.

(e) After the mass centers are determined and entered into the computer, a series of injections are made to determine the relative ion ratios and the ultimate mass spectrometer sensitivity to the derivatized chloramphenicol. The series of injections should as well as tissue samples spiked at levels comparable to that expected from the incurred samples to be examined.

(f) The sample cleanup from the quantitative portion of procedure 5.022, is suitable for GC/MS work. There appears to be a slight matrix effect on apparent sensitivity, i.e., a 200 pg injection of standard produces somewhat more intense peaks than injection of an aliquot containing a supposedly similar amount of chloramphenicol from either a fortified or metabolically incorporated muscle sample.

(g) Mass spectral confirmation of chloramphenicol, six ions are monitored. The presence of five ions with proper ratios for at least four of the ions are required for confirmation. Two ions, m/z 466 and 468, are from the molecular ion cluster and indicate the presence of two chlorine atoms in the molecule. These two ions must be present for confirmation. Two other chlorine-containing ions are monitored, at m/z 358 and 360. These ions contain one chlorine atom. (If very low levels of chloramphenicol are injected, m/z 360 occasionally disappears into the background noise, and cannot be observed.) Two non-chlorine containing portions of the molecule, at m/z 304 and 322 are also monitored. All observed ions from incurred samples must have retention times equivalent to that found for standard chloramphenicol (± 10 percent). The ratio of ions should be similar to that found for standard chloramphenicol. Typical ratios are:

<u>IONS</u>	<u>Relative Ratio</u>
446/468	1.3
358/466	0.15
358/360	2.7
304/466	1.25
322/466	0.75

The 304/466 and 322/466 ratios are very dependent on the ion source tuning and more critically, ion source cleanliness. As the source becomes more contaminated, these ratios will increase. Confirmation, however, is based upon a comparison of ion ratios from a tissue sample with those found for standard material, and will not be adversely affected.

(h) Sample injection - It is desirable to inject a quantity of derivatized chloramphenicol equivalent to approximately five times the minimum confirmable quantity. This quantity is normally injected into the mass spectrometer in a volume of 5 microliters or less. Therefore, the final extract volume from the analytical assay may have to be adjusted by concentration or dilution to achieve the desired level.

5.024 Screening Procedure for Pentachlorophenol in Liver Tissues

5.024A. Theory

This procedure is based on the extraction of pentachlorophenol (PCP) into cyclohexane and removal of interfering compounds by a strong acid treatment. Quantitative transfers are eliminated by the addition of pentabromoethylbenzene (PBEB) as a reference compound. The PCP and PBEB are quantitated directly on a gas liquid chromatograph equipped with an electron capture detector. The screen has a usable range from 50 to 5000 ppb.

5.024B. Apparatus

- (a) Culture tubes, screw cap, teflon liner, 16 x 125 mm, 20 x 150 mm and 25 x 150 mm.
- (b) Replacement caps for culture tubes, teflon liner, size: 15-415 and 24-410.
- (c) Heating blocks, Multi-Blok Heaters, or equivalent, size to accept 20 mm and 25 mm tubes.
- (d) Reciprocating horizontal shaker, Eberbach 600, or equivalent.
- (e) Solvent dispensers, Brinkmann Dispensette, or equivalent, sizes 0.4 to 2 ml, 1.0 to 5.0 ml and 5 to 25 ml.
- (f) Measuring syringes, sizes 100 ul, 200 ul and 500 ul.
- (g) Test tube support, sizes 15-16 mm, 18-20 mm, and 22-25 mm O.D.
- (h) Disposable pipets, Pasteur type, 9" length.
- (i) Gas liquid chromatograph, Hewlett-Packard 5840, or equivalent, equipped with Ni-63 electron capture detector, 3' x 2 mm id glass column packed with 1% SP-1240DA on 100-120 mesh Supelcoport.

5.024C. Reagents

- (a) Cyclohexane: Burdick and Jackson, or equivalent, pesticide grade.
- (b) 2-Propanol: Burdick and Jackson, or equivalent, pesticide grade.
- (c) Toluene: Burdick and Jackson, or equivalent, pesticide grade.
- (d) H_2SO_4 : Reagent grade.
- (e) 12 M H_2SO_4 : Add 66 ml of con. H_2SO_4 to 33.0 ml H_2O .
- (f) Pentabromoethylbenzene: 98% pure, Aldrich Chemical Company.
- (g) Pentachlorophenol: Pesticide Reference Standards Section, EPA Research Triangle Park, N.C.

5.024D. Preparation of Standards

- (a) Pentachlorophenol (PCP)
 - (1) Stock A (1mg/ml)

Weigh out a 100 ± 5 mg sample of PCP and transfer to a 100 ml volumetric flask. Dissolve sample and make up to volume with toluene. Store in refrigerator and prepare fresh solutions every 3 months.

(2) PCP Working Standard-Range 50-500 ppb (2 $\mu\text{g/ml}$)

Dilute 1 ml of stock A to 500 ml with 2-propanol, using a volumetric flask. Store in refrigerator and prepare fresh monthly.

(3) PCP Working Standard-Range 100-1000 (4 $\mu\text{g/ml}$)

Dilute 2 ml of stock A to 500 ml with 2-propanol, using a volumetric flask. Store in refrigerator and prepare fresh monthly.

(4) PCP Working Standard-Range 500-5000 ppb (20 $\mu\text{g/ml}$)

Dilute 10 ml of stock A to 500 ml with 2-propanol, using a volumetric flask. Store in refrigerator and prepare fresh monthly.

(b) Pentabromoethylbenzene (PBEB)

(1) Stock B (1.2 mg/ml)

Weigh 120 ± 5 mg sample of PBEB and transfer to a 100 ml volumetric flask. Dissolve sample and make up to volume with toluene. Store in the refrigerator and prepare fresh stock every 3 months.

(2) Internal Reference Solution - 50-500 ppb (600 ng/ml)

Dilute 0.5 ml of stock B to 1000 ml with 2-propanol, using a volumetric flask. Store at room temperature and prepare fresh solutions monthly.

(3) Internal Reference Solution - 100-1000 ppb (1.20 $\mu\text{g/ml}$)

Dilute 1.0 ml of stock B to 1000 ml with 2-propanol, using a volumetric flask. Store at room temperature and prepare fresh solutions monthly.

(4) Internal Reference Solution - 500-5000 ppb (6.0 $\mu\text{g/ml}$)

Dilute 5.0 ml of stock B to 1000 ml with 2-propanol, using a volumetric flask. Store at room temperature and prepare fresh solutions monthly.

5.024E. Sample Preparation

Blend a 50 g liver sample in a 1 quart blender jar until a homogeneous mixture is obtained. Transfer the homogenized sample to a 6" x 4.5" plastic bag. Heat seal the bag and store at -20°C until assayed.

5.024F. Extraction and clean-up

(a) Range 50-500 ppb

(1) On the day of the assay, thaw the frozen liver packet to room temperature. Cut a corner from the packet and squeeze a 2.0 ± 0.1 g sample into a 150 x 25 mm glass screw cap tube, held vertical inside a beaker, on a top loading balance.

(2) Using a Brinkman dispenser, add 5 ml of 12M H_2SO_4 to each tube. After each addition, immediately cap the tube with a teflon lined cap and hand shake vigorously to prevent large aggregates from forming. Smaller lumps on the tube walls and at the surface will be dispersed by the end of the digestion period.

(3) After completing the acid additions, transfer the tubes to a heating block and heat for 60 ± 5 minutes at $97 \pm 3^\circ\text{C}$. At 10 minute intervals briefly hand shake each tube. *Caution:* Hot H_2SO_4 is very corrosive and appropriate personal protective clothing should be worn. This includes lab coat, rubber gloves and apron, goggles and face shield. For added protection shake the glass tubes inside a plain, round bottom, 100 ml polypropylene centrifuge tube, behind a safety shield.

(4) At the end of the heating period, cool the tubes to room temperature in a basin of water. To the cooled digestates, add 10 ml of cyclohexane and 2 ml of appropriate Internal Reference Solution, using the Brinkman dispensers. Cap tubes and shake for 15 ± 2 minutes on a mechanical shaker.

(5) At the end of the extraction period, allow phases to separate and transfer a 4 ± 1 ml aliquot of the upper phase to a 16 x 125 mm screw cap glass culture tube, using a disposable glass transfer pipet.

(6) Add one ml of concentrated H_2SO_4 to the cyclohexane aliquot, using a Brinkman dispenser. Cap the tubes with teflon lined caps and shake on a mechanical shaker for 2 minutes.

(7) At the end of the shaking period, allow the tubes to stand for 30 minutes. Transfer an aliquot of the upper phase to a gas chromatograph autosampler vial. Cap the vials and store in a freezer. Analyze within 24 hours.

(b) Range 100-1000 ppb

As in (a) above, except use 20 ml of cyclohexane instead of 10 ml as in step (a)(4).

(c) Range 500-5000 ppb

As in (a), range 50-500 ppb, except at the end of step (a)(4), transfer a 1 ml aliquot of the cyclohexane layer to a 16 x 125 mm glass tube and mixed with 10 ml of cyclohexane. Use a 4 ± 1 ml aliquot of this solution in step (a)(5).

5.024G. Standard Curve Preparation

(a) Prepare G.C. standards by mixing 10 ml of blank matrix cyclohexane (see note 1), 2 ml of Internal Reference Solution-Range 50-500 ppb and 50, 100, 200 and 500 μl of PCP Working Standard-Range 50-500 to yield 50, 100, 200 and 500 ppb equivalent PCP in 2 grams tissue, respectively.

(b) Chromatograph standards with samples of interest.

(c) Measure the peak heights of PCP and PBEB. Calculate the ratio of PCP to PBEB.

$$y = \frac{\text{height of PCP peak}}{\text{height of PBEB peak}}$$

(d) using a linear regression, construct a standard curve of peak height ratio vs. equivalent ppb PCP.

The equation is $y = mx + b$

Where: y = ratio peak height PCP to peak height PBEB.

x = ppb PCP

m = slope

b = y-intercept

The correlation coefficient (r) should be ≥ 0.990

- (e) Using the regression slope and intercept, the PCP concentration for each incurred sample may be calculated.

Preparation of Blank Matrix Cyclohexane:

NOTES: 1) The matrix has an enhancing effect on the PCP response necessitating the preparation of standard in a cyclohexane extract of blank liver. Prepare the extract by using the extraction and clean-up procedure with the following modifications:

Step (1) Weigh 8.0 g.

Step (2) Add 20 ml of 12M H₂SO₄.

Step (4) Transfer digest to 125 ml separatory funnel, add 40 ml cyclohexane and 8.0 ml 2-propanol. Shake for 2 minutes.

Step (5) Transfer all cyclohexane to 25 x 150 mm tube.

Step (6) Add 5 ml concentrated H₂SO₄ and shake for 10 minutes.

Step (7) Transfer cyclohexane to clean flask and use for preparation of GC standards.

2) The 50-500 ppb range response curve will be the same as the 10-1000 or 500-5000 curves if the working PCP standards and Internal Reference Solutions are equivalent after appropriate dilutions. To check the concentrations of standards in each range, add 200 µl of PCP Working Standard, 2ml of 50-500, 100-1000 and 500-5000 ppb ranges respectively. The CG quantitation of these solutions, expressed as the ratio of PCP to PBEB, should vary by less than 5%. Care needs to be taken to adjust the Brinkman Dispensette so that the same volume of Internal Reference Solution is delivered for all ranges.

5.024H. GC Conditions

Injector port temperature: 180°C

Column temperature: 160-180°C

Detector temperature: 275°C

Flow Rate: 30 cc/min

Sensitivity: 66 pg aldrin 50% full scale at 2 minutes

Auto Sampler: 2 µl

(b) Column

Glass: 3 ft x 2 mm, glass on column injection

Packing: 1% SP-1240DA on 100/120 mesh Supelcoport

Use a very small amount of H₂PO₄ treated glasswool at the exit end only. Condition the column overnight at its maximum temperature of 189°C.

(c) Retention times

PCP: 2-3 minutes

PBEB: 4-6 minutes

(d) Performance

Column should be reconditioned when peak broadening is noted or after 200 sample injections. The column can be adequately reconditioned by removing the first 6 cm of packing, scrubbing the walls with a glasswool plug and replacing

the packing with fresh material. Condition the column by elevating the temperature to 180°C overnight. The column may be hooked to the detector during this conditioning period.

5.024I. Calculations

(a) PCP determination

- (1) Measure peak height of PCP and PBEB and express as the ratio of PCP to PBEB
- (2) Determine the amount of PCP by substituting the PCP/PBEB ratio into the standard curve equation

Where $y = mx + b$
 x = PCP in ppb
 m = slope of standard curve
 y = PCP/PBEB peak height ratio
 b = intercept of standard curve

- (3) Correct for recovery by dividing the calculated amount of PCP in step (2) by the % recovery times 100.

(b) Recovery

- (1) With each set, run 2 blank tissues fortified with 100 ml of PCP Working Standard of appropriate range.
- (2) Calculate the amount recovered using the equation in (a);(2).
- (3) Percent recovery is determined by dividing the fortification level into the amount recovered.

5.025 Pentachlorophenol Gas Chromatograph/Mass Spectrometry Confirmatory Procedure

5.025A. Theory

This is a method to confirm the presence of pentachlorophenol in extracts from beef, pork, and chicken liver tissue by quadrupole GC/MS. A capillary column is used for separation with electron impact ionization for detection/confirmation. The method is sensitive enough to detect 10 ng of methylated pentachlorophenol. The action level for pentachlorophenol is 50 ppb, thus any violative samples must be re-extracted from an 8 or 10 gram tissue sample.

5.025B. Apparatus

- (a) Instrument—Hewlett Packard 5992 GC/MS with capillary column capability, or equivalent.

GC Conditions and Parameters:

Column—25 meter OV-101 Fused Silica Capillary

Injector—160°C

GC Oven—110°C for 1 minute, then programmed to 250°C at 15°C/minute, then held for 1 minute

Vent Delay—1 minute

Solvent Elution Delay—2 minutes

Column Pressure—16 psi

Mass Spectrometer Conditions: Because the confirmatory method for the methylated derivative of pentachlorophenol is performed on a Hewlett Packard 5992 GC/MS/COM system, operator control of mass spectrometry parameters is limited to keeping track of the change in the auto tune voltage and in the absolute and relative ion intensities of the calibrating compound and/or standard (derivatized pentachlorophenol). Thus, the source should be cleaned when the autotune voltage rises by 400 eV over that found for a freshly cleaned source, or when the minimal confirmatory quantity has increased by a factor of four. The analysis is run at the autotune voltage or 200 eV above the autotune voltage. The major problem in confirming a sample is the erratic presence of extraneous compounds which have mass spectral responses in the retention time window of the analyte. The presence of these matrix-related materials vary dramatically from sample to sample, and prevent a definitive limit of confirmation from being determined.

- (b) Vials, conical bottom, 200 ul

- (c) Dry nitrogen delivery system

- (d) See 5.024B.(a)—(h)

5.025C. Reagents

- (a) See 5.024C.(a)—(g)

- (b) Hexane—Burdick and Jackson, or equivalent

- (c) Dry nitrogen

(d) Diazomethane—Prepare using directions supplied with Diazald (Aldrich Chemical Company, P.O. Box 355, Milwaukee, Wisconsin; catalog #D2, 800-0). Use of the Diazald Kit (catalog #Z10, 0250-0) is strongly advised. Diazomethane can be successfully and safely stored for 2-4 weeks, in ultra-cold freezers, provided the sample is properly sealed to keep out moisture.

5.025D. Derivatization Procedure

- (a) Perform steps 5.024F. (a)(1)-(a)(7), except use a 200ul conical bottomed vial.
- (b) Blow sample extract to dryness with dry nitrogen.
- (c) Add 100-200 ul of "active" diazomethane and let sit for 15 minutes.
- (d) Carefully evaporate solvent using a gentle stream of nitrogen.
- (e) Wash down the vial walls with hexane and repeat step (d).
- (f) Dilute residue to an appropriate volume with hexane. (This volume is based upon instrument sensitivity and an injection volume of no more than 5 ul into the GC.)

5.025E. Acquisition Parameters and Analysis of Selected Ion Monitoring Data

Monitor six ions from methyl pentachlorophenyl ether for 50 milliseconds by GC/MS to confirm the presence of pentachlorophenol. The monitored ions include two ions from the molecular ion cluster, two ions from the $(M-CH_3)^+$ cluster and two ions from the $(M-COCH_3)^+$ cluster. The monitored ions are:

Molecular ion cluster— m/z 278 and 280

$(M-CH_3)^+$ cluster— m/z 263 and 265

$(M-COCH_3)^+$ cluster— m/z 235 and 237

Confirmation of a positive sample requires the presence of the six ions. At least three ion ratios relative to m/z 278, including the m/z 280/278 ratio, must be within ± 15 percent of the ratio found for the same ions relative to m/z 278 in samples spiked at approximately the same level. The retention time should be within ± 5 percent of that found for standard material.

5.026 Detection of Styrene in Meat by GC/MS Headspace Analysis

5.026A Theory

Styrene can be detected at levels 1-10 ppb by gas chromatographic/mass spectrometric (GC/MS) analysis of headspace vapors from tissues exposed to styrene. The analysis is performed on a GC/MS system equipped with a 30 m OV-1 capillary column.

Styrene is soluble in organic solvents and volatilizes rapidly in air. Precautions must be taken to prevent contamination of laboratory environment and sample during handling, preparation, and analysis.

Pure styrene must be kept in a working hood. All materials coming into contact with pure styrene must remain in the hood.

All items which are to come into direct contact with tissue samples must be solvent rinsed, (do not place plastic parts in solvents), and dried in an oven at 100°C. Air dry items with plastic or wood parts.

Metal or ceramic surfaces are preferred for sample preparation. These are easily solvent wiped and dried quickly.

Samples cannot be handled with protective gloves or bare hands. A suitable barrier when handling samples can be provided by aluminum foil sheets.

Samples must remain frozen until sealed for headspace analysis.

5.026B Apparatus

- (a) Hewlett Packard 5992 GC/MS system, or equivalent.
- (b) 30 meter, OV-1 (dimethyl silicone) capillary column, or equivalent.
- (c) Vials — 10 ml crimp top, Teflon sealed.
- (d) Syringes — 1.0 ml (0.1 ml graduated)
100 ul (10 ul graduated)
10 ul (1 ul graduated)
- (e) Forceps — medium, sharp pointed, 4-6" long.
- (f) Scalpels and blades — No. 3 industrial scalpel with No. 10-X blades.
- (g) Cotton tipped applicator sticks — 6", wooden.
- (h) Paper or cotton towels - lint free.
- (i) Metal pan.
- (j) Ceramic bench top protector.

5.026C Reagents

- (a) Methylene Chloride — Burdick and Jackson, or equivalent.
- (b) Methanol — Burdick and Jackson, or equivalent.
- (c) Hexane — Burdick and Jackson, or equivalent.

(d) Acetone — Burdick and Jackson, or equivalent.

(e) Styrene — Fisher Scientific Co., or equivalent.

5.026D Determination

(a) Preparation of apparatus and glassware:

(1) Solvent rinse all vials, syringes and tools which will come directly into contact with tissue samples. Rinse all syringes using vacuum to draw solvents through syringe barrel. Use the following sequence for solvent cleaning: methylene chloride, methanol, hexane, and acetone.

(2) Saturate a cotton or paper towel with solvent and wipe all surfaces with solvents as sequenced above.

(3) Air dry items with plastic or wooden parts. Dry syringes by continuously drawing a vacuum through the syringe barrel. Dry other items in an oven at 100°C for 15 minutes.

(4) Place all cleaned items in a metal tray, and allow them to cool before preparing samples.

(5) Rinse a 100 ul syringe in the solvents as sequenced in (a)(1) above. Remove plunger and place barrel and plunger in a 100°C oven for 30 seconds. Prolonged heating of the syringe may cause the seals to deteriorate and leak.

(b) Preparation of control and standard:

(1) Prepare a control blank by sealing an empty 10 ml crimp top, Teflon sealed vial.

(2) Prepare a 10 ppm standard by sealing 0.1 ul of pure styrene in a 10 ml vial.

(c) Preparation of sample:

(1) Prepare samples by trimming 3-5 g of tissue from exterior of sample. Seal trimmings in a 10 ml vial. Interior trimmings can be taken to determine degree of saturation.

(2) Fortify an extra tissue sample with 10 ul of styrene.

NOTE: It has been observed that meat tissue absorbs up to 99 percent of the styrene with which it is fortified.

(d) Gas Chromatography/Mass Spectrometry:

(1) Set GC parameters as follows:

injection port temp = 160°C

oven temp 1 = 70°C for 1 minute

oven temp 2 = 240°C, programmed at 16°C/minute and hold for 1 minute.

solvent elution time = 1.0 minute

vent delay = 60 seconds

column pressure = 15 PSI

retention time of styrene = 1.6 minutes

(2) Run GC/MS system through a "dry run" analysis, without injecting, to assure minimal column bleed at monitored masses ($m/z = 51, 52, 77, 78, 103, \text{ and } 104$).

(3) Placed sealed vials in 100°C oven for 15 minutes prior to injection and analysis.

(4) Inject 100 μl of headspace from "control blank" 10 ml vial.

(5) Repeat (a)(5).

(6) Inject 100 μl of headspace from a tissue sample.

(7) Repeat (a)(5).

(8) Inject 100 μl of empty syringe to check syringe.

(9) Repeat (a)(5).

(10) Repeat (6), (7), and (8) above.

5.026E MS Interpretation and GC parameters

(a) Monitor masses 51, 52, 77, 78, 103, 104 in SIM mode with a dwell time of 200 milli seconds.

(b) Set EM voltage at 200 eV above autotune voltage.

Using the above parameters, a positive finding is indicated by a peak for styrene at all monitored masses with a retention time of 3 to 4 minutes.

5.027 Detection of Gasoline in Canned Meat by GC/MS Headspace Analysis

5.027A. Theory

The canned product is cooled in a freezer before opening, because canned meats do not usually have enough headspace for conveniently taking a saturated vapor sample. Fat is transferred to a 10 ml glass vial which is then sealed with aluminum foil and capped with a Teflon coated, rubber septum and aluminum cover. The vial is heated at 100°C for 15 minutes and a 100 ul injection is made using a gas-tight syringe on a gas chromatograph equipped with a flame ionization detector (FID).

5.027B Apparatus

- (a) Gas Chromatograph: Hewlett-Packard Model 5830, or equivalent, equipped with a flame ionization detector.
- (b) Stainless steel column 6' \times 4 mm ID glass column (80 ml/min N₂ flow rate), packed with G.P. 5 percent SP-1200/1.75 percent Benton 34, on 100/120 mesh Supelcoport.
- (c) Nitrogen, hydrogen, and compressed air sources at 20 ml/min (or 80 ml/min, if glass column is used), 10 ml/min, and 100 ml/min respectively.
- (d) Glass vials, 10 ml, with suitable septums and covers.
- (e) Gas-tight syringe: 100 ul, Hamilton (1710-N), or equivalent.
- (f) Syringes: 10 ul and 100 ul.
- (g) Forced draft oven, or other heating device, set at 100°C.
- (h) Volumetric flask: 10 ml.

5.027C. Reagents

- (a) Gasoline — (regular or unleaded).
- (b) Petroleum Ether—(pesticide grade).
- (c)(d)(e) Ortho, meta and para xylenes — (reagent grade).
- (f) Ethyl benzene — (reagent grade).
- (g) Working Standards.

Gasoline — 1:10, gasoline/petroleum ether, V/V.

Ortho xylene — 1:10, o xylene/petroleum ether, V/V.

Meta xylene — 1:10, m xylene/petroleum ether, V/V.

Para xylene — 1:10, p xylene/petroleum ether, V/V.

Ethyl benzene — 1:10, ethyl benzene/petroleum ether, V/V.

5.027D. Determination

- (a) Place canned meat sample in a freezer for one hour.
- (b) Transfer approximately one gram of fat from the surface of the solidified meat mass inside the can to a 10 ml glass vial.
- (c) Seal the vial with aluminum foil, a Teflon coated, rubber septum, and the aluminum cover cap, in that order.
- (d) Run approximately 1 ppm and 10 ppm standards in parallel with sample by adding 10 ul and 100 ul respectively of 1:10 gasoline/petroleum ether to 1.0 gram of clean fat in each of two vials and performing steps 5.027D.(e) and (f), below.
- (e) Heat the sample in the sealed vial in a forced draft oven, or other suitable heating device, at 100°C for 15 minutes.
- (f) Using a 100 ul gas-tight syringe, inject a 100 ul headspace sample immediately from the heated vial into a GC, which is equipped with one of the two columns described in 5.027B. (b), above, under the following conditions:

Injection temperature — 100°C.

Column temperature — 75°C.

FID temperature — 200°C.

Nitrogen carrier gas flow rate — 20 ml/min (Stainless Steel, 6' × 1/8" ID column) or 80 ml/min (Glass, 6' × 4 mm ID column).

Hydrogen flow rate — 10 ml/min

Compressed air flow rate — 100 ml/min

The best technique for injection is by "pumping" the syringe six times at moderate speed, filling the syringe slowly and making the injection within 30-90 seconds after the removal of the vial from the oven. Delay of injection results in drastically reduced response.

Retention time for all lower boiling fractions is approximately 25 minutes. Do not perform next injection until these fractions are eluted.

- (g) Inject 10 ul each of the o, m, and p xylenes, and ethyl benzene working standards.

5.027E. Evaluation

Quantitation for gasoline is not possible because instrument response is not linear. Repeatability with the same tissue is good. Positive identification is made by comparing the retention times and relative peak heights of o, m, and p xylenes, and ethyl benzene. The lower detection limit depends upon the sensitivity of the FID, but will be in the vicinity of 0.1 ppm gasoline.

5.028 Analysis of Lysergic Acid Diethylamide and Phencyclidine in Tissue by TLC

5.028A. Theory

The drugs of interest are extracted from an alkaline tissue sample with ethyl acetate. The organic phase is then shaken with dilute hydrochloric acid. The pH of the acid is adjusted with base and extracted with chloroform. The chloroform layer containing the drugs is then removed and spotted on two separate TLC plates, developed and visualized with the appropriate spray reagents.

5.028B. Apparatus

- (a) 50 ml polypropylene screw-top disposable centrifuge tubes
- (b) Mechanical horizontal shaker (240 cycles/min or equivalent)
- (c) Vortex test tube mixer
- (d) Centrifuge capable of 3000 rpm.
- (e) Lab timer
- (f) 15 ml glass centrifuge tube
- (g) Water bath 15-20°C
- (h) Repipet dispensers, 30 ml and 5 ml
- (i) 10 ml class A volumetric flasks
- (j) 100 ml volumetric flasks
- (k) 100 ul syringe (glass)
- (l) E. Merck 60F-254 silica gel plates

5.028C. Reagents

- (a) Ethyl acetate, distilled in glass
- (b) 50% NaOH, reagent grade
- (c) Granular sodium sulfate
- (d) 0.5N HCL, reagent trade
- (e) Chloroform, distilled in glass
- (f) LSD — lysergic acid diethylamide — 0.02 mg/ml in ethyl acetate
- (g) PCP — Phencyclidine HCL — 0.117 mg/ml in ethyl acetate
- (h) Acetone — Distilled in glass
- (i) Methanol — Distilled in glass

(j) Iodoplatinate (potassium) — for alkaloids and other organic nitrogen compounds. Mix 3 ml 10% hexachloroplatinic (IV) acid solution with 97 ml water and add 100 ml 6% potassium iodide solution in water; freshly prepare the reagent before use.

(k) Dimethylaminobenzaldehyde-hydrochloric acid — for indole derivatives. Dissolve 1 g 4-dimethylaminobenzaldehyde in 50 ml 36% hydrochloric acid and add 50 ml ethanol.

5.028D. Determination

1. To 10 grams of homogenized meat add one ml of 50 percent sodium hydroxide in 50 ml polypropylene centrifuge tube.
2. Add 5 grams of sodium sulfate to the mixture and 30 ml of ethyl acetate.
3. Shake the solution on a horizontal shaker for 10 minutes.
4. Centrifuge the tube at 3000 RPM for 5 minutes.
5. Pour off the ethyl acetate upper layer into another 50 ml polypropylene centrifuge tube.
6. Remove any oily residue that transfers over with a pasteur pipet and discard.
7. Add 5 ml of 0.5N Hydrochloric acid, shake for 3 minutes using a horizontal shaker and then centrifuge at 2000 RPM for 3 minutes.
8. Aspirate off the ethyl acetate layer (upper layer).
9. Transfer the 5 ml of 0.5 ml hydrochloric acid solution to a 15 ml graduate glass centrifuge tube.
10. Cool the solution in a water bath for 5 minutes and then add one ml of a 50 percent sodium hydroxide solution, and cool again for 3 minutes.
11. Agitate by vortexing for 30 seconds.
12. Cool the solution again in a water bath for two minutes.
13. Add 100 ml of chloroform, to the solution (alkaline) shake manually for three minutes.
14. Centrifuge for 5 minutes at 200 RPM.
15. Aspirate off half of the aqueous alkaline layer and discard:
 - (a) With a 100 ul syringe, remove two 40 ul aliquot portions of the chloroform layer and spot separately on different silica gel plates. Spot a detectable amount of LSD and PCP separately on each plate.
 - (b) Develop the plates in the following solvent system: Acetone: Methanol (80:20). Saturate the tank prior to use.
 - (c) After development air dry the plates and spray for LSD using P-dimethylaminobenzaldehyde. Spots will be violet on a yellow plate. Spray for PCP using Iodoplatinate. Spots will be brown on a yellow plate.

(Note: Spray lightly — do not over spray).

Rf LSD — 0.33

Rf PCP — 0.25

5.029 Procedure for the Analysis of Lasalocid in Bovine Liver by HPLC

5.029A. Theory

Ten gram aliquots of ground liver are extracted with 40 ml acetonitrile. A 23.5 ml portion of the extract is washed with hexane and the acetonitrile layer is evaporated to dryness under nitrogen at 55-65°C. One ml of water saturated with the HPLC mobile phase and 2 ml of the HPLC mobile phase are added to the residue. After vortexing and centrifugation, a portion of the organic (top) layer is removed, and an aliquot is analyzed by HPLC on two 25 cm Partisil PXS 10/25 (Whatman) columns. The effluent is monitored by fluorescence with excitation set at 310 nm and emission at 440 nm.

The peak height is determined, and the quantity of Lasalocid is calculated from an external standard curve run on the same day.

The fortification is done on acetonitrile enzyme deactivated liver sample because of Lasalocid's instability on spiking into liver. Lasalocid is stable in livers from dosed animals.

5.029B. Apparatus

1. 80 ml glass centrifuge tubes.
2. Polytron — Brinkman Industries equipped with small blending heat or equivalent.
3. 50 ml polypropylene tubes (corning).
4. Centrifuge — Damon/IEC Model HN-s equipped with six place fixed rotor or equivalent.
5. N-Evap, Model 111 — Organomation Assoc. Inc. or equivalent.
6. Maxi Mix — Themolyne, Sybron corp.
7. HPLC Chromatograph — Waters Associates, Model 244, or equivalent, with Schoeffel FS 970 LC Fluorometer, detector, Wisp 710B Sample processor, and a strip chart recorder Perkin Elmer recorder Model 056 1001.
8. Pre-Injection Column — Whatman partisil PXS 10/25 (10 micron microparticulate Silica — 25 cm 4.6 mm i.d.).
9. Analytical Column — Two Whatman partisil PXS 10/25 in series.
10. 100 μ l Syringe
11. Usual glassware

5.029C. Reagents

1. Hexane — distilled in glass (Burdick and Jackson)
2. Methanol — distilled in glass (Burdick and Jackson)
3. Tetrahydrofuran — distilled in glass (Burdick and Jackson)
4. Acetonitrile — distilled in glass (Burdick and Jackson)
5. Ammonium Hydroxide — ACS reagent grade
6. Standard Lasalocid (mw 612.80). Source: Animal Health Research, Hoffman LaRoche.

Decontamination of apparatus and glassware

1. Meat Grinder — All parts of the grinder which come in contact with the tissue sample should be washed with hot soapy water, rinsed, air-dried, rinsed with ethyl acetate, and air-dried. The hot soapy water wash should be repeated between sets of samples expected to contain different levels of Lasalocid.

2. Polytron — After homogenizing a sample, wipe the remaining liver off the shaft using a clean paper towel and rinse the shaft successively in water 2×50 ml (in a 250 ml centrifuge bottle), 50 ml ethyl acetate, and 50 ml acetonitrile with polytron set at medium speed for 10-15 seconds for each rinse.

5.029C. Determination

(a) Preparation of HPLC mobile phase

1. Prepare the following solvent mixture, adding the components in order to decreasing volume percents:

Mixture A:	Hexane	—	810 ml
	Tetrahydrofuran	—	150 ml
	Methanol	—	30 ml
	Ammonium Hydroxide	—	10 ml

Mix thoroughly in separatory funnel and let stand until upper phase is clear (about one hour). Empty lower phase and discard. Reserve upper phase. Process three volumes (three separatory funnels) simultaneously.

2. The mobile phase is stable for a minimum of one week.

(b) Preparation of water saturated with HPLC mobile phase.

1. Mix 50 ml water (deionized, distilled) with 100 ml HPLC mobile phase in a 250 ml separatory funnel. Shake vigorously for 30-40 seconds.

2. Let sit until lower layer (water) is clear. Remove lower layer immediately prior to use.

3. Prepare fresh each day.

(c) Preparation of Lasalocid standard solutions for standard curve and sample fortification.

1. 140 ug/ml Lasalocid — Weigh exactly 72.66 mg lasalocid sodium salt standard into a 500 ml volumetric flask. Add THF to dissolve lasalocid and dilute to volume with THF.

2. 70 ug/ml Lasalocid — Dilute 50 ml of standard (1) to 100 ml with tetrahydrofuran.

3. 35 ug/ml Lasalocid — Dilute 25 ml of standard (1) to 100 ml with tetrahydrofuran.

For standard curve dilute 100 ul of each of the standard to 4 ml with mobile phase representing 1.4, 0.7 and 0.35 ppm respectively.

Store stock solutions in refrigerator in stoppered volumetric flasks sealed with parafilm. Solutions may be kept for five months. Each week, decant a fresh supply of each solution into screw-capped culture tubes, seal with parafilm, and store at room temperature for use in sample fortification and generation of daily standard curves.

(d) Preparation of fortified samples

1. Weigh 10.0 ± 0.05 g of partially defrosted liver sample into a clean 80 ml glass centrifuge tube. Select a blank liver sample for a second control and three levels of fortification.

2. Add 40 ml acetonitrile to each weighed sample and homogenize for 15-30 seconds.

3. Fortify the homogenized blank samples according to the following table:

Fortification Level (ppm)	Standard Lasalocid	$\mu\ell$ of Fortifying Solution/10g Sample
1.4	5.029C.(c)1.	100
0.7	5.029C(c)2.	100
0.35	5.029C.(c)3.	100

4. Homogenize each sample again for 15-30 seconds.

5. Centrifuge for 10 minutes at 2000-2500 rpm.

6. Using a 25 ml graduated cylinder, transfer 23.5 ml aliquot into a 50 ml screwcapped polypropylene tube.
(This represents $\frac{1}{2}$ of the sample extract assuming 10 g liver to contain approximately 7 ml water.)

7. Add 20 ml hexane.

8. Shake vigorously for 15-20 seconds, with venting.

9. Centrifuge at 1500-2000 rpm for 10 minutes at room temperature.

10. Aspirate hexane layer and discard.

11. To remove interfering fat, repeat steps 7-10.

12. Evaporate acetonitrile layer to less than 0.5 ml under nitrogen using N-evap. at 45°-60°C. *Do not overdry.*

13. Add 1 ml water saturated with mobile phase.

14. Vortex for 15-20 seconds.

15. Add 2.0 ml HPLC mobile phase and vortex for 15-20 seconds with the screw cap on.

16. Transfer the mixture into 15 ml stoppered glass centrifuge tube with *tapered bottom*.

17. Centrifuge at 1500-2000 rpm for 10 minutes.

18. Transfer about 200 $\mu\ell$ *clear* upper layer into a capped plastic vial for HPLC analysis.

19. With each set of samples, process a control liver, reagent blank, and a 0.7 ppm fortified liver to determine recovery.

(e) HPLC analysis

Follow SOP to HPLC with the following exceptions:

(i) Do not filter or de-gas mobile phase

(ii) Do not filter samples

(iii) Each day, transfer only enough mobile phase mixture for a daily run from the amber gallon bottle into the HPLC solvent bottle. Cover solvent bottle with aluminum foil.

1. Set the fluorescence spectrophotometer as follows:

Excitation:	310 nm	Slit: 8 nm
Emission:	440 nm	Slit 8 nm
Sensitivity:	10, 3 or 1 as needed to keep Lasalocid peak on scale	

PM Gain:	Norm
Response:	Norm
Mode:	Norm
Zero Suppression:	Off

2. Equilibrate the entire system with mobile phase until a stable baseline is obtained (about 30-45 minutes). If the column is new, equilibrate with mobile phase until a constant retention time and fluorescent response for Lasalocid are obtained (about 24 hours).

3. Measure the flow rate at the beginning of each day's samples and recheck periodically throughout the day.

4. Using Wisp 710B sample processor, inject (in duplicate) 20 μ l portions each of sample extracts and mixed standards.

5. Retention time for Lasalocid is about 6.5 minutes at a flow of 2.0 ml/min. Chart speed should be about 0.5 cm/min. Allow 10 minutes between injections.

6. With each set of samples, inject three different Lasalocid standards before the set, and the same three standards after the set. The concentration of Lasalocid injected should be above, below and approximately the same concentration as expected to be present in the final dilution of the samples to be assayed.

ppm Lasalocid in Liver	Expected Concentration Lasalocid per Final Dilution of Liver Extract
0.35	35 ug/ml
0.7	70 ug/ml
1.4	140 ug/ml

7. If the HPLC system is not to be used for eight hours or more, flush the entire system with hexane.

Note:

(a) Use of Automatic Ingegration Devices.

Laboratory instrumentation that automatically provides peak area or peak heights can be used in the method. The technique used should be common for all the samples and the calculation should reflect techniques used in height or area.

(b) Retention Time or Volume of Lasalocid.

The retention volume or time will shift based on the history of the column. The standards are run at the start and end of a series and are used to determine responses as well as retention time.

Variation in retention time is also influenced by the time marking process either manually or automatically. This also is resolved by inspection of multiple standards.

5.029D. Calculations

Calculation of Lasalocid in Sample: Using area data or peak height from 1.40, 0.70 and 0.35 ppm standard lasalocid injections, construct a linear standard curve based on the formula $y = mx + b$, where x is the peak area or height and y is ppm. Calculate regression coefficient and standard error of estimate for these data using the following formulas:

1. Slope
$$m = \frac{n \sum xy - \sum x \sum y}{n \sum x^2 - (\sum x)^2}$$

2. Intercept
$$b = \frac{(\sum y - m[\sum x])}{n}$$

3. Regression Coefficient
$$r = \frac{\sum xy}{(\sum x^2 \cdot \sum y^2)^{1/2}}$$

4. Standard Error of Estimate
$$s = \left\{ \frac{1}{n - 2} \left[\frac{\sum y^2 - (\sum xy)^2}{\sum x^2} \right]^{1/2} \right\}$$

Calculate recovery from a spiked sample included in every set. Correct sample values for recovery.

5.030 QUANTITATION OF DECOQUINATE IN BEEF LIVER, KIDNEY AND MUSCLE TISSUES

This method utilizes a fluorometric analysis for the quantitative determination of decoquinatate (ethyl 6 — (decyloxy) — 7 — ethoxy — 4 — hydroxy — 3 — 1 quinolinecarbonylate. A gas chromatographic qualitative identification procedure is used because the fluorometric procedure is not specific.

5.030A. Theory

Beef tissue is homogenized in methanol-chloroform, centrifuged, and a known portion of the supernate transferred to a separatory funnel. After addition of metaphosphoric acid, decoquinatate is extracted into chloroform, separated from interfering materials by Florisil column chromatography, and quantitatively determined by spectrophotometric fluorescence detection. A portion of the final extract is derivatized with diazomethane — methanol for gas chromatographic confirmation. Confirmation is required because Necquinatate and Buquinolactate are also detected by this procedure. If either of these compounds are determined to be present by confirmation, quantitative values can be obtained by substituting that compound for Decoquinatate in the procedure as written.

Safety Precautions

Diazomethane is toxic and potentially explosive. Preparations, methylations, and evaporations must be done in a hood. Avoid glass joints, etched or scratched glassware, and sharp edges. Diazomethane solutions must be stored in a freezer and must not be exposed to direct sunlight or strong artificial light. Prepare only the amount of diazomethane necessary, take precautions and use safety shield, and face shields.

5.030B. Apparatus

- (a) Grinder
- (b) Balance, top loader, (Mettler P1200, or equivalent)
- (c) Kinematica tissue blender, or equivalent
- (d) Sorvall RC-5B centrifuge with GSA rotor, or equivalent
- (e) Polypropylene centrifuge bottles, 250 ml capacity, equipped with screw caps.
- (f) Perkin Elmer 650-10S Fluorescence Spectrophotometer, or equivalent, equipped with 10 mm sample cell.
- (g) Chromatographic column 10 mm × 300 mm, equipped with stopcock.
- (h) Graduated glass centrifuge tubes, 15 ml capacity. equipped with stoppers.
- (i) Graduated cylinders, 25 ml, equipped with stoppers.
- (j) 5, 10, 50, 100 and 250 ul syringes and micropipettes.
- (k) 250 ml and 125 ml separatory funnels.
- (l) Volumetric flasks, pipettes and other glassware as required.
- (m) Diazomethane generator.
- (n) Tracor 222 gas chromatograph (or equivalent) with electron capture Ni 63 detector and 6 foot glass column. I.D. 1/4" packed with 1.5% OV 17 and 1.95% OV210 on Chromosorb WHP.
- (o) N-Evap analytical evaporator.

5.030C. Reagents

- (a) Methanol, Burdick and Jackson pesticide grade.
- (b) Chloroform, Burdick and Jackson pesticide grade.
- (c) Decoquate reference standard (Hess and Clark Laboratories).
- (d) Anhydrous calcium chloride.
- (e) 5% metaphosphoric acid solution in water.
- (f) Methanol-Chloroform (4 + 1).
- (g) Elution solvent — dissolve 10 g anhydrous CaCl_2 in 1 liter redistilled methanol. Let stand 24 hours and filter.
- (h) Decoquate standard solutions.

1. Stock solution 200 ug/ml—weigh 20 mg decoquate ref. standard. Dissolve in CHCl_3 and dilute to volume in a 100 ml volumetric flask and mix well.

2. Working solution 10 ug/ml — Pipet 5 ml stock solution into a 100 ml volumetric flask and dilute to volume with CHCl_3 .

3. Fluorescence reference solution 0.2 ug/ml — Pipet 2 ml working solution into 100 ml volumetric and dilute to volume with elution solvent.

All standard solutions must be stored under refrigeration and are stable one month.

- (i) Florisil — Supelco
- (j) 1 N NaOH
- (k) Benzene, Burdick and Jackson pesticide grade.
- (l) Diazomethane — Prepare diazomethane using the following proportions of reagents for reaction mixture.

1. 300 mg Diazold (n-Methyl-n-Nitroso-p-Toluene-Sulfonamide)

2. 2.0 ml ethyl ether

3. 2.0 ml 60% KOH

4. 2.0 ml Dowanol, carbitol, 2-(2-ethoxy-ethoxyl-1 ethanol) — Place 10 ml ether into collection tube submerged in an ice bath. Generate diazomethane by bubbling nitrogen through reaction mixture and collect for 5 minutes. Store ethyl ether solution of diazomethane in the freezer. Solution should be strong yellow and is stable for ≤ 1 week.

5.030D. Standard Curve

Weigh 50 g MeOH-CHCl_3 (4 + 1) solution into each of four 250 ml separatory funnels. Add 0, 5, 15 and 25 ul stock solution containing 0, 1, 3 and 5 ug decoquate, respectively. Proceed with determination beginning "Add 100 ml 5% metaphosphoric acid", adding entire CHCl_3 extract to column. Construct standard curve by plotting peak height against ug decoquate/ml eluate (ppm).

5.030E. Gas Chromatography

- (a) Tracer 222, or equivalent
- (b) Column — 6 ft. \times 1/4" i.d., glass column packed with 1.5% OV-17 and 1.95% OV-210 on Chromosorb WHP
- (c) Detector ^{63}Ni electron capture
- (d) Typical operating conditions
 - (1) Column temperature — 235°C
 - (2) Detector temperature — 305°C
 - (3) Injector temperature — 275°C
 - (4) Flow-rate — 20 ml per minute 95/5, Argon/Methane, for column. 60 ml per minute 95/5, Argon/Methane, for purge.
 - (5) Electrometer sensitivity attenuation — 4.
 - (6) Recorder — 0.25 inches/minute.
 - (7) Normal retention time for decoquinatate is 10-12 minutes.

5.030F. Florisil Column Preparation

- (a) Accurately weigh 0.40 ± 0.01 g florisil.
- (b) Position a glass wool plug at the bottom of a clean glass column 10 mm \times 300 mm, equipped with a stopcock.
- (c) Close stopcock and pour approximately 10 ml CHCl_3 into the column.
- (d) Add the Florisil slowly and wash down the sides of the column with an additional 5 ml CHCl_3 . With a clean glass stirring rod, gently stir the Florisil layer to remove air bubbles.
- (e) Drain off CHCl_3 until it is just above the Florisil layer. Do not permit the column to run dry.

5.030G. Determination

- (a) Weigh 20 ± 0.20 g tissue into a 250 ml capacity centrifuge bottle.
- (b) Add 80 ± 0.50 g MeOH-CHCl_3 (4 + 1) and blend 60 ± 0.5 seconds at medium setting (Apparatus(c)).
- (c) Centrifuge 5 minutes at 2000 rpm.
- (d) Decant and weight 50 ± 0.5 g of supernate (equivalent to 10 g tissue) into 250 ml separatory funnel.
- (e) Add 100 ± 1 ml 5% metaphosphoric acid and gently invert 50 times over a 2 minute period. (Vigorous agitation will cause an emulsion).
- (f) Allow phases to separate 10.0 minutes. drain and save the CHCl_3 layer in a 25 ml graduated cylinder equipped with a stopper.
- (g) Add 10 ml CHCl_3 to the separatory funnel, invert and allow to separate as before.

(h) Combine CHCl_3 extracts, add 2.0 ml MeOH and dilute to 25 ml with CHCl_3 . Mix thoroughly.

(i) Depending on expected decoquinat content, pipet 5, 10, or 25 ml of extract (equivalent to 2, 4, or 10 g of tissue respectively) onto the chromatography column. Run extract through column with stopcock fully open. Stop flow with Florisil barely covered with extract. Discard solvent. (Pipetted volumes of 5 ml for expected 2 ppm; 10 ml for expected 1 ppm; and 25 ml for expected 0.5 ppm will keep final readout within standard curve.)

(j) Wash column with exactly 10 ml MeOH (pipet) and discard washings.

(k) Elute sample with exactly 15 ml (pipet) of elution solvent and collect in tube graduated to 15 ml and equipped with a stopper. Collect exactly 15 ml.

(l) With fluorescence reference standard in the cell and slit widths at 2 mm (or adjusted to minimize background interference and give optimum response), set the fluorometer activation wavelength at 270 nm and emission wavelength at 390 nm, and adjust the microphotometer controls to give an $80 \pm 2\%$ reading of full scale.

(m) Determine fluorescence of sample by scanning emission wavelength from 330 to 530 nm. Measure peak height and determine micrograms of decoquinat from standard curve.

5.030H. Confirmation

(a) Transfer 10.0 ml of sample eluate to a 125 ml separatory funnel containing 15 ml distilled water.

(b) Extract once with 10 ml CHCl_3 and once with 5 ml CHCl_3 using same extraction and separation parameters described previously. Combine chloroform extracts in a 15 ml evaporation tube.

(c) Evaporate chloroform just to dryness on N-Evap and take up residue in 0.20 ml methanol.

(d) Add 1 ml of freshly prepared diazomethane ether solution, vortex five seconds and stopper. Allow to stand at room temperature for one hour in subdued light.

(e) Gently evaporate the diazomethane ether with a nitrogen stream. Add 0.10 ml benzene and gently invert ten times. (Vigorous agitation will cause emulsions.)

(f) Inject 5 μl onto GC and compare to retention time of standard.

5.030I. Calculations

$$\text{Recovery} = \frac{\text{ppm Decoquinat found}}{\text{ppm Decoquinat added}} \times 100$$

$$\text{ppm Decoquinat} = \text{ppm found} \times \frac{15 \times 25 \times 2}{\text{ml CHCl}_3 \text{ extract used} \times 20}$$

where: ml CHCl_3 are from step 5.030G(i).

5.031 DETERMINATION OF TETRACYCLINE IN ANIMAL TISSUES BY HPLC

5.031A. Theory

A method is described for the extraction and quantitation of oxytetracycline, tetracycline and chlortetracycline in liver, kidney and muscle tissue of food producing animals and fowl.

Tissue is extracted with 0.05M H_3PO_4 and 0.01M Na_2EDTA . Protein is precipitated by addition of 50 percent trichloroacetic acid followed by heat treatment. The clarified extract is acidified with concentrated HCl and tetracyclines are converted to anhydro forms by heat treatment. After pH adjustment to 3.4 ± 0.1 with sodium citrate, anhydrotetracyclines are extracted with methylene chloride. The methylene chloride extract is evaporated to dryness and the residue dissolved in methanol. Quantitation is by HPLC. The procedure has been examined quantitatively for tetracyclines in swine, cattle and turkey.

The tetracycline antibiotics have very widespread use. Analysts must keep the varying tolerances in mind when assaying samples. See chart below.

Oxytetracycline (Hydrochloride)					Chlortetracycline					Tetracycline	
TOLERANCE					TOLERANCE					TOLERANCE	
ppm					ppm					ppm	
SPECIES	M	L	K.	F	SPECIES	M	L	K	F	SPECIES	ET
Chickens	1	1	3	1	Chickens	1	1	4	1	Calves	0.25
Turkeys	1	1	3	1	Turkeys	1	1	4	1	Swine	0.25
Swine	0.1 ET				Ducks	1	1	4	1	Sheep	0.25
Cattle	0.1 ET				Swine	1	2	4	0.2	Chickens	0.25
Beef & Dairy Calves—	0.1 ET				Calves	1	1	4	1	Turkeys	0.25
Non lactating Dairy Cattle —					Cattle	0.1	0.1	0.1	0		
0.1 ET					Milk —	0					
					Eggs —	0					
M = Muscle					L = Liver					K = Kidney	
										F = Fat	
										ET = Edible Tissues	

5.031B. Apparatus

(a) Centrifuge — Damon/IEC CU-5000 with IEC 240 head (Damon/IEC Division, Needham Heights, MA 02194), or equivalent.

(b) N-Evap — Model #111 or 112 (Organomation Associates, Northborough, MA 01532), or equivalent.

(c) Vortex mixer — any suitable model.

(d) Tissue homogenizer — Model SDT (Tekmar Company, Cincinnati, Ohio, 45222), or equivalent.

(e) Electric shaker — Eberbach Model #5850 (Eberbach Corporation, Ann Arbor, MI 48106), or equivalent.

(f) Pipettes, measuring, 10 ml delivery volume.

(g) Pipettes, volumetric, 10 ml delivery volume.

(h) Funnel, powder.

- (i) Automatic transfer pipets 15 ml, 2 ml (Fisher Scientific 13-683-10 series), or equivalent.
- (j) pH meter, Corning Model 125, or equivalent.
- (k) Polypropylene tubes, 100 ml (Curtin Matheson 084-186), or equivalent.
- (l) Culture tube with screw cap, teflon liner, 50 ml (VWR 60827-599), or equivalent.
- (m) Centrifuge tube, 15 ml glass stoppered (VWR 20922-029), or equivalent.
- (n) Filter paper, Reeve Angel #802, 12.5 cm.
- (o) Volumetric Flasks, low actinic, 100 ml and 50 ml.

(p) Liquid Chromatograph — Waters Associates model 244, or equivalent, with model 440 Absorbance detector, WISP 710B Sample processor and model 730 Data module, or equivalent. Conditions and operating parameters: Column C₁₈ reverse phase packing 40 mm i.d. × 25 cm., 10 μ M (R. E. Gourley Company, 8763 Contee Road, Laurel, Maryland 20810). Also marketed by Bodman Chemical Co., as Cleer Sil C₁₈. No other columns are acceptable.

Mobile Phase

Preparation of stock mobile phase buffer — Into a 1 L beaker, weigh 105.1 ± 0.1 g liquid diethanolamine. Dilute to ca 700 ml with distilled water, add 7.44 ± 0.01 g disodium EDTA · 2H₂O. Add magnetic stirring bar and stir until EDTA is dissolved. Titrate on pH meter with syrupy phosphoric acid to pH 7.3 ± 0.1 . Quantitatively transfer to 1 L volumetric flask and dilute to volume with distilled water. Stock 1 M buffer is stable indefinitely at room temperature.

Preparation of Working Mobile Phase — To prepare mobile phase, place 50 ml of stock buffer into stoppered 1 L graduate mixing cylinder, dilute to ca 600 ml with distilled water, then add 140-200 ml isopropanol (to produce final isopropanol concentration of 14-20 percent v/v), and dilute to 1 L with distilled water. Mix, filter, and degas before use. Final concentration of isopropanol needed to produce resolution of all components in standard mixture varies slightly from column to column with the isocratic solvent used in the method. 14-16 percent isopropanol is usually sufficient for the 1.5 ml/min column flow used, but must be determined experimentally for each column. The 14 percent isopropanol concentration solvent produces separation of all components in mixed standard, requiring approximately 25 minutes. The order of elution for the major components is: anhydrotetracycline (ATC), B-apo-oxytetracycline (Apo-OTC), anhydrochlorotetracycline (ACTC). For optimal elutions of individual tetracyclines 14 percent isopropanol is used for anhydrotetracycline, 16 percent isopropanol for B-apo-oxytetracycline and 20 percent isopropanol for anhydrochlorotetracycline. The above solvent concentrations should produce elution times of 6-8 minutes for individual anhydrotetracyclines.

Column flow — 1.5 ml/min (6000 A pump)

Chart speed — 0.5 cm/min (Data module)

Noise rejection — 50 (Data module)

Absorbance — 254 nm (440 UV Detector)

Area rejection — 100 (Data module)

Peak width — 5 (Data module)

Sensitivity — 0.2 AUFS (440 UV Detector)

Note: Column should be washed after the last run for ca 30 minutes with 50 methanol: 50 ethanol saturated with Na₂ EDTA (500 ml MeOH, 500 ml EtOH, add Na₂ EDTA and mix for 20 minutes. Filter and degas solvent).

Column Sensitivity/Acceptability — Column must be checked upon receipt for acceptable resolution. Newly delivered columns should be first flushed for 30-60 minutes with the MeOH:EtOH wash solution at 1.5 ml/minute, then equilibrated with mobile phase. Inject standard mixture containing 200 ng of each anhydrotetracycline. Monitor chromatogram at 0.02 AUFS. All components must be resolved satisfactorily. Peaks of all three tetracycline derivatives must be symmetrical with minimal tailing.

5.031C. Reagents

- (a) Methanol — Distilled in glass — Burdick and Jackson, or equivalent.
- (b) Isopropanol — Distilled in glass — Burdick and Jackson, or equivalent.
- (c) Methylene Chloride — Distilled in glass — Burdick and Jackson, or equivalent.
- (d) Disodium EDTA — ACS grade 0.01 M and 0.10 M.
- (e) Phosphoric Acid — ACS grade 0.05 M and concentrated.
- (f) Hydrochloric Acid — ACS grade.
- (g) Trichloroacetic Acid — ACS grade, 50% aqueous.
- (h) Diethanolamine — ACS grade (Fisher or J. T. Baker).
- (i) Sodium Citrate — ACS grade.
- (j) Tetracycline standards — Highest purity available, Sigma Chemical Company, St. Louis, MO 63178.

Note: Distilled water used in making all reagents and buffers must be HPLC grade, either bottled or Millipore — Milli-Q purified, having low organic content and a specific resistance of at least 10 megohms/cm to avoid problems with extraneous interferences.

(k) Standards

1. Stock Solutions

(i) Parent tetracycline stock solutions (stable one month in refrigerator). Into separate 100 ml low actinic volumetric flasks, weigh 10 mg each of tetracycline, oxytetracycline and chlortetracycline. Dissolve and dilute to volume with methanol, (100 ug/ml).

(ii) Anhydrotetracycline stock solutions (stable one month in refrigerator). Into a 100 ml low actinic volumetric flask weigh 100 mg anhydrotetracycline standard. Dissolve and dilute to volume with methanol (1 mg/ml). Prepare apo-oxytetracycline and anhydrochlorotetracycline stock solutions as above.

2. Working Standards

(i) Into individual clean 50 ml low actinic volumetric flasks, pipet 1 ml of each stock anhydrotetracycline standard. Dilute to volume with methanol (20 ug/ml).

(ii) Into a clean 50 ml low actinic volumetric flask pipet 2 ml of stock anhydrotetracycline standard. Dilute to volume with methanol (40 ug/ml).

(iii) Into a clean 50 ml actinic volumetric flask pipet 3 ml of stock anhydrotetracycline standard. Dilute to volume with methanol (60 ug/ml).

(iv) Mixed anhydrotetracycline standard — Into a clean 100 ml low actinic volumetric flask, pipet 1 ml of each individual anhydrotetracycline stock solution. Dilute to volume with methanol (10 ug/ml).

(v) Prepare mixed parent tetracycline standard as above in (iv). Anhydrotetracycline standard solution can be prepared if commercially prepared standard is not available. Pipet 1.0 ml of mixed parent tetracycline standard (10 ug/ml) into 50 ml glass centrifuge tube. Remove methanol with a stream of nitrogen. Add 15 ml 0.05 M H_3PO_4 and 15 ml

Na₂EDTA. Carry through the steps 1-7 of the sample clean up procedure beginning at "add 2 ml concentrated HCl". Dissolve residue in 1 ml methanol. (Conc. = 10 ug/ml ACTC and ATC, 5 ug/ml Apo—OTC). Store in cool dark place. Stable two weeks or more in refrigerator.

5.031D. Determination

(a) Preparation of Standard Curve — Inject 10 ul each of standard anhydrotetracyclines at 20 ug/ml, 40 ug/ml, and 60 ug/ml to construct a linear standard curve. Plot peak height vs ng injected.

(b) Sample Preparation — Tissue should be prepared according to instructions in USDA Laboratory Guidebook, section 1.002B. Tissue should be kept frozen at all times until analyzed.

Note: Tetracycline is not stable under direct overhead fluorescent light. Carry out extraction cleanup steps with overhead fluorescent lights turned off.

(c) Weigh 10 g of ground tissue into a 100 ml polypropylene centrifuge tube. Use a blank tissue as control. Use a second blank sample and fortify at 4 ppm by adding 400 ul of parent stock solution (100 ug/ml). Allow tissue to stand at room temperature for 15 minutes.

(d) Add 15 ml 0.04M H₃PO₄ and 15 ml 0.01M Na₂EDTA. Add 2 ml 50% TCA.

(e) Blend with tissue homogenizer at medium speed (ca. 10,000 rpm) for 1 minute. Let stand 15 minutes at room temperature.

(f) Heat in boiling water bath for 10 minutes. Mix briefly with tissuemizer.

(g) Centrifuge 10 minutes at 2500 rpm.

(h) Chill sample in ice bath until fat congeals. Note: The muscle tissue samples tend to gel after long chilling.

(i) Filter through qualitative (Reeve Angel # 802) filter paper into 50 ml glass tube with teflon-lined screw cap. Briefly rinse the filter paper with distilled water (ca 3-4 ml), adding rinse to collected filtrate.

(j) Add 2 ml concentrated HCl and heat for 10 minutes in boiling water bath.

(k) Chill immediately 5 minutes in ice bath.

(l) Add 4 g solid sodium citrate to each tube.

(m) Shake for 10 minutes using mechanical shaker until sodium citrate dissolves.

(n) Check pH. Adjust pH to 3.3 — 3.5 by addition of more sodium citrate or backtitrate by dropwise addition of concentrated HCl.

(o) Add 15 ml methylene chloride, cap the tube and shake 10 minutes on mechanical shaker at low speed.

(p) If no emulsions are present, centrifuge for 10 minutes at 1500 rpm. If emulsions are present, centrifuge for 20 minutes at 1500 rpm.

(q) Remove upper layer by vacuum aspiration and discard.

(r) Draw up 10 ml methylene chloride layer using 10 ml volumetric pipette and transfer to a 15 ml centrifuge tube.

(s) Evaporate methylene chloride to dryness using N-Evap with a stream of dry nitrogen and bath temperature at 55° — 60°. Temperature should not exceed 60°.

Note: Care should be taken during evaporation to dryness step. Do not allow this step to proceed unattended. Significant loss may occur if residue is allowed to over-dry.

- (t) Dissolve the residue in 200 ul methanol for injection. The standard or usual injection is 10 ul.

Note: Samples from injection sites often contain very high levels of tetracycline. Visual inspection will indicate orange-colored extracts. Dilute such extracts further with methanol until extract assumes pale lemon yellow color. Reduce volume injected to 5 ul or less.

Stopping Points

Any step can be used as a stopping point, with the exception of the step after the addition of HCl and adjustment of pH (step n). It is necessary that methylene chloride should be added as soon as possible. It is not desirable to allow tetracycline to remain in pH 3.3 — 3.5 (see step n) too long to prevent further epimerization of the compound. Once the final evaporation is completed, samples should be injected on the HPLC as soon as possible. Do not hold samples overnight in methanol before injection.

5.031E. Calculations

1. Using the data from 20 ug, 40 ug, 60 ug standard injections construct a linear standard curve based on formula $y = mx + b$ where x is peak area and y is ug. Calculate regression coefficient and standard error of estimate for this data.

Regression Coefficient

$$r = \frac{\sum xy}{\sqrt{(\sum x^2)(\sum y^2)}}$$

Standard error of estimate

$$s = \sqrt{\frac{\sum y^2 - \frac{(\sum xy)^2}{\sum x^2}}{n - 2}}$$

2. Using regression slope and intercept, calculate the tetracycline concentration (y) for each sample from measured peak area.

3.
$$\frac{\text{ug Tetracycline concentration}}{\text{Sample weight} \times \text{dilution factor}} = \text{ppm tetracycline}$$

4. Oxytetracycline ppm should be multiplied by 2. Oxytetracycline forms equal quantities of alpha and beta apoxytetracycline during the acid conversion step. Methylene chloride extracts only the beta-isomer. Because of this fact, determined values for oxytetracycline must be multiplied by 2 to produce the correct answer.

Notes:

1. Both parent tetracyclines and anhydrotetracyclines (oxytetracycline epimerization is structurally inhibited) begin epimerizing the instant they are placed in solution. Epimerization is accelerated by heat, light and aqueous solution.
2. Although standards in methanol and low actinic glassware are fairly stable under refrigeration, epimerization eventually requires preparation of fresh standards.
3. Epimers are not quantitated in this procedure, because standards are not available and the amount of epimer generated during the assay is generally 5% or less of the individual tetracyclines present in samples.

4. Columns used in this assay are a specially prepared C18 reverse phase. No other commercially available columns are suitable for trace level tetracycline monitoring.

5.032 GAS CHROMATOGRAPHIC PROCEDURE FOR TRIAZINE RESIDUES IN FOWL AND BEEF FAT

5.032A. Theory

A gas chromatographic procedure using a capillary column and nitrogen-phosphorus detector has been developed for management of residues of propazine, terbutylazine, atrazine and simazine in fat. The fat sample is dissolved in hexane and partitioned against acetonitrile. The acetonitrile is evaporated and the residue taken up in water-acetonitrile and passed through a reverse phase mini column. Triazines are eluted with methanol-water and partitioned into methylene chloride from aqueous solution. The methylene chloride is evaporated to dryness and the residue taken up in nonane for GC analysis on a Durawax DX-4 column.

5.032B. Apparatus

(a) Perkin-Elmer Sigma 1 gas chromatograph, or equivalent with Grob type splitless capillary injector and nitrogen-phosphorus detector, automatic sampler (see appendix for sensitivity requirements).

(b) J&W Durawax DX-4 fused silica column, 30 m \times 0.25 mm i.d. (available from J&W Scientific, Inc., Applied Science or Perkin-Elmer Corp).

(c) Electronic leak detector, J&W GC Protector, Gow-Mac Gas Leak Detector (Supelco), or equivalent for detecting hydrogen leakage from GC column fittings.

(d) Oxygen scrubber — Alltech Associates Oxy-Trap, or equivalent.

(e) Disposable C₁₈ extraction columns, 3 ml/500 mg, J. T. Baker Chemical Company PN 7020-3.

(f) 75 ml reservoir and connector union for (e), J. T. Baker PN 7120-3.

(g) 50 ml glass centrifuge tube Pyrex PN 8240.

(h) 15 ml conical glass centrifuge tube. Fisher PN-05-538-20A, or equivalent.

(i) 60 ml separatory funnel.

(j) Repipet dispensers for solvents (6).

(k) Organomation N-Evap.

(l) Calculator capable of linear regression analysis.

(m) Vacuum manifold for eluting C18 cartridges (See Appendix).

(n) Steam bath

(o) 250 ml beakers

(p) 100, 250, 500 microliter syringes.

5.032C. Reagents

(a) Acetonitrile: Distilled in glass, or equivalent.

(b) Hexane: Distilled in glass, or equivalent.

- (c) Water HPLC grade (Millipore, Burdick and Jackson, etc.)
- (d) Methanol-water (80:20 v/v): Use distilled in glass grade methanol and HPLC grade water.
- (e) 0.1M NaCl: Reagent grade.
- (f) Methylene Chloride: Distilled in glass, or equivalent.
- (g) Hexadecane: Reagent grade.
- (h) Nonane: Distilled in glass, or equivalent.
- (i) Analytical Triazine Standards — EPA, Research Triangle Park, NC 27711.
- (j) Ethylacetate.
- (k) Isooctane — Distilled in glass, or equivalent.

5.032D. Preparation of Stock Standards

1 mg/ml Stock solutions are prepared by accurately weighing 25 mg of each triazine into separate 25 ml volumetric flasks. Dissolve and bring to volume with ethylacetate, except for simazine. For simazine use acetone as the solvent. Store in glass and refrigerate. Prepare new stock solutions every three months.

5.032E. Preparation of Working Standards

(a) Fortification Standards:

1. Pipet 1 ml of each of the four stock standards into a single 100 ml volumetric flask. Bring to volume with isooctane (10 ug/ml).
2. Pipet 10 ml of the combined 10 ug/ml standard into a 50 ml volumetric flask and bring to volume with isooctane (2.0 ug/ml). Label this standard "40 ppb fortification standard." Transfer to a stoppered glass tube and refrigerate.
3. Pipet 5.0 ml of the combined 10 ug/ml standard into a 50 ml volumetric flask and bring to volume with isooctane (0.1 ug/ml). Label this standard "20 ppb fortification standard." Transfer to a stoppered glass tube and refrigerate.
4. Pipet 5.0 ml of the combined 10 ug/ml standard into a 100 ml volumetric flask and bring to volume with isooctane (0.5 ug/ml). Label this standard "10 ppb fortification standard," and refrigerate.
5. Prepare new working standards every three months.

(b) Gas Chromatography Standards

1. To 1.0 ml of the 40 ppb fortification standard add 2.0 ml nonane and label it "40 ppb GC standard."
2. To 1.0 ml of the 20 ppb fortification standard add 2.0 ml nonane and label it "20 ppb GC standard."
3. To 1.0 ml of the 10 ppb fortification standard add 2.0 ml nonane and label it "10 ppb GC standard."

— Sample Preparation

Render fat as outlined in 5.001F.(1) or extract as outlined in 3.005.

5.032F. Determination

(a) Extraction

1. Weigh 5.00 g of melted fat into a 50 ml glass centrifuge tube. Select blank fat and weigh two samples. Use one as a control blank and fortify the other with 100 ul of the 20 ppb fortification standard.

2. Condition, by gravity flow, C_{18} columns by washing with: (1) 3 ml methanol, (2) 3 ml water.

3. Add 10 ml hexane to the samples and mix. Transfer to a 60 ml separatory funnel, rinsing sample container with 2 additional 10 ml portions of hexane. Beef fat samples may need to be warmed in a water bath to effect solution, and should remain above 25°C during extraction.

4. Add 10 ml acetonitrile to the separatory funnel, shake for 1 minute. Allow layers to separate and collect acetonitrile fraction in 250 ml beaker.

5. Repeat with 3×10 ml portions of acetonitrile. Combine all extracts with first extract.

6. Remove acetonitrile on a steam bath. Monitor closely. Do not allow beaker to remain on the steam bath once the acetonitrile has evaporated.

7. Dissolve the residue in 5 ml acetonitrile. Add 100 ml water.

8. Transfer the solution to the prewetted C_{18} cartridge. Maintain vacuum at 10-12 inches mercury.

9. Wash beaker with 5 ml water and add to column. Do not allow column to go completely dry. Break vacuum just after the last drops of water exit the column.

10. Elute the column with 8 ml methanol-water (80:20, v/v). Collect in 50 ml centrifuge tubes.

11. Transfer the eluate to a 60 ml separatory funnel. Rinse the tube with 2×8 ml portions of 0.1 M NaCl solution and add these rinses to the separatory funnel.

12. Add 12 ml methylene chloride to the separatory funnel, shake for 1 minute. Allow layers to separate and collect methylene chloride fraction in a 15 ml conical glass centrifuge tube. Retain aqueous phase.

13. Add 10 ul hexadecane to the methylene chloride and evaporate the methylene chloride to the residual hexadecane under a stream of nitrogen at 35°C.

14. As methylene chloride evaporates repeat extraction of aqueous phase with 2×12 ml portions of methylene chloride.

15. Combine with the first portion when tube capacity permits. Dissolve the residue in 290 ul nonane. (290 ul nonane + 10 ul hexadecane = 300 ul final volume).

(b) Gas Chromatography (Typical operating conditions — will need adjustment from column to column.)

Column: 30 meter \times 0.25 mm i.d. Durawax DX-4 fused silica capillary column operating conditions:

(a) Injection port temperature — 195°C

(b) Detector temperature — 220°C

(c) Initial oven temperature — 130°C

- (d) Initial time — 2 minutes
- (e) Temperature program — 40°C/min.
- (f) Second oven temperature — 195°C
- (g) Second time — 35 minutes
- (h) Temperature program — 40°C/min.
- (i) Final time — 15 minutes
- (j) Final time — 15 minutes
- (k) Injector backflush time — 0.75 minutes
- (l) Attenuation — 1 or 2
- (m) Chart speed — 2 mm/sec
- (n) Integrator sensitivity — 400; 100
- (o) Carrier gas (hydrogen) velocity — approximately 60 cm/sec @ 205°C
- (p) Detector make-up gas (nitrogen) flow rate — 50 ml/min
- (q) Detector hydrogen pressure — 14 psi
- (r) Detector air pressure — 26 psi
- (s) Injection volume — 5 ul

See Appendix for additional information on setup, maintenance and performance standards for instrument and column. The final temperature program to 205°C. is for eluting late components which otherwise would appear in later chromatograms.

5.032G. Calculations

Using either peak area or peak height from the 10, 20, and 40 ppb standard injections construct a linear standard curve based on the formula $y = mx + b$, where x is the peak area or height, and y is concentration in ppb. Calculate the correlation coefficient (r) and the standard error of estimate (S_{yx}) for the standard curve. Calculate the recovery from the fortified control sample included with each sample set. Correct sample values for recovery by using the average of the last ten recovery values (ten day moving average).

5.032H. Appendix

- (a) Lifetime of column is substantially increased by:

1. Use of hydrogen as carrier gas — low O_2 content plus a degree of regenerative power because of its reductant nature.
2. Use of oxygen scrubber on carrier gas line — change regularly, every three cylinders.
3. Maintenance of column below 500°C when not in use.

4. Septum changes only after column and injection port are at 50°C or below.
5. Keeping column and injection port at 50°C or below for one hour after septum change.

Failure to observe (4) and (5) for a Carbowax or Durawax column can result in rapid or even instantaneous column failure.

(b) Column performance and sensitivity can be optimized by:

1. Insertion of column 5 cm into injection port liner.
2. Insertion of column almost (1-2 mm) to flame end of the jet tip.

Hint: Insert column and if jet tip glows yellow, back off column until its color just disappears.

3. Maintenance of clean injection port linear, silanized and periodically rinsed with methanol and pipe cleaner and resilanized. Change liner every 50 samples.

4. Optimized detector and make-up gas flows. Values given in this procedure are for a Perkin-Elmer Sigma 1 only at a linear velocity of 60 cm/sec. Optimum conditions for other instruments need be arrived at empirically. Change in the hydrogen carrier gas flow rate substantially affects the nitrogen-phosphorus detector and requires appropriate changes in the air and hydrogen detector gas flow rates to compensate. Consequently it is advisable to set the lowest column flow rate compatible with 20-25 minute total analysis time, then optimize detector parameters and maintain these values for the life of the column.

(c) Sensitivity, Resolution

In order to adequately detect and resolve the triazines from the tissue matrix, the following conditions should be met:

1. Minimum sensitivity — 1 nanogram atrazine injected ($0.333 \text{ ng/ul} \times 3 \text{ ul}$) produces 100% FS deflection at 1% noise (i.e., $S/N = 100$). Typically S/N is 150-200 for 1 ng atrazine.
2. Resolution — atrazine theoretical plate count = 60,000 plates.

(d) Interferences

There are two significant interference peaks in the chromatograms of tissue extracts. The first elutes between propazine and terbutylazine. It is a tissue component and from MS data may be dioctylphthalate. The second peak elutes immediately after simazine and is a reagent impurity. Although both peaks can be resolved from the triazines, care must be taken not to confuse these peaks with the triazines. The chromatography is sufficiently reproducible that retention time differences of 0.1 — 0.2 min are significant.

(e) Peak Measurement

If possible, peak area should be used for quantitation. Because of the narrow bore of the column, peaks are slightly retarded and broadened compared to a solvent standard. Peak height will give an apparent low result. Use of peak height will probably result in better reproducibility because it ignores widening of the peak base widths in real samples due to low level interferences. On the other hand, peak area by integrator is much more convenient as it is not necessary to keep all peaks on scale for measurement. The analyst may wish to use a one point (20 ppb) calibration of the integrator to quantitatively screen all samples and then use a three point standard curve by peak height to formally quantitate only violative samples.

(f) Stopping Points

The ideal situation is to start a set of samples at the beginning of the day, then use an automatic sampler to inject the samples on the GC overnight. If the analysis cannot be completed in one day, acceptable stopping points are 5.032 F(a), 4, 9, 10. Care should be taken not to allow the samples to remain on the steam bath or N-Evap evaporator unattended as extended heating greatly increases the loss of the triazines.

5.033 RAPID SCREEN FOR LEVAMISOLE RESIDUES IN TISSUE

5.033A. Theory

Levamisole is extracted from an alkaline tissue sample with ethyl acetate. The organic phase is shaken with dilute hydrochloric acid. The pH is adjusted with base followed by extracted with chloroform. The levamisole concentration is measured by GLC using a Flame Photometric detector.

5.033B. Apparatus

- (a) 50 ml polypropylene screw-top disposable centrifuge tubes
- (b) Mechanical horizontal shaker (240 cycles/min, or equivalent)
- (c) Vortex test tube mixer
- (d) Centrifuge capable of 3000 rpm.
- (e) Laboratory timer
- (f) 15 ml glass centrifuge tube
- (g) Repipet dispensers, 30 ml and 5 ml
- (h) 10 ml class A volumetric pipets
- (i) 100 ml volumetric flasks
- (j) 100 ul syringe (glass)
- (k) RP 5840 GLC, or equivalent, equipped with a Flame Photometric detector (6" × 2 mm I.D. glass column packed with 3 percent OV-17 on 80/100 mesh Gas Chrom Q)
- (l) MP 5840 GLC, or equivalent, equipped with Flame Ionization detector (30 m OV-101 capillary column)

(The conditions below are for a specific instrument. Conditions will vary from instrument to instrument and should be adjusted to give 30 percent to 50 percent full scale deflection for the 0.1 ppm levamisole standard.)

For FPD Detector:

Col. temp: 250°C
Inj. temp: 260°C
Detector temp: 240°C
Flow Rate: 60 ml/min

For FID Detector:

Col. temp: 70°C — 320°C
programed at 30°/min
Detector temp: 250°C
Flow Rate: 1 ml/min

5.033C. Reagents

- (a) Ethyl acetate, distilled in glass
- (b) 50% KOH, reagent grade
- (c) Granular sodium sulfate
- (d) 0.5N HCl, reagent grade.
- (e) Chloroform, distilled in glass

(f) Levamisole HCl (Aldrich Chemical Company)

(g) 2, 5 bis (4-pyridyl) 1, 3, 4-thiadiazole (Aldrich Chemical Company)

5.033D. Preparation of Standard Solutions

(a) Stock levamisole solution: Dissolve 10.0 mg of levamisole in a 100 ml volumetric flask and dilute to mark with water (100 ug/ml).

(b) Working levamisole solution: Pipette 10.00 ml of stock standard and dilute to 100 ml with water (10 ug/ul). To fortify at 0.1 ppm, add 100 ul to 10 g sample.

(c) Stock internal standard: Dissolve 15.0 mg of 2, 5 bis (4-pyridyl) 1, 3, 4-thiadiazole in approximately 5 ml of 1N HCl in a 100 ml volumetric flask. Mix well and dilute to 100 ml with water (150 ug/ml).

(d) Working internal standard: Pipette 10.00 ml of stock solution and dilute to 100 ml with water (15 ug/ml). To fortify add 100 ul to each 10 g sample.

5.033E. Determination

(a) Weigh 10 g of blended frozen tissue into a 50 ml polypropylene screw-top centrifuge tube, and allow to thaw to room temperature. Add 100 ul of a 15 ug/ml solution of the internal standard, then add 5 g Na_2SO_4 . Spread evenly over surface of tissue. (See Note 1)

(b) Add 1 ml of 50 percent KOH and 30 ml ethyl acetate to the above tube. Shake hard for 10 seconds, let stand 15 minutes, then place on mechanical shaker for 10 minutes.

(c) Centrifuge at 3000 rpm for 5 minutes.

(d) Pour 20.0 ml of the ethyl acetate into another 50 ml centrifuge tube. Do not transfer any oily layer. Add 5 ml 0.5N HCl to the ethyl acetate and shake for 2 minutes.

(e) Centrifuge at 2000 rpm for 2 minutes.

(f) Draw off and discard the ethyl acetate with a pasteur pipette connected to a vacuum and transfer the acid layer to a 15 ul glass, conical bottom centrifuge tube. Add 1 ml 50 percent KOH. Vortex 20-30 seconds to mix and allow to cool to room temperature.

(g) Add 100 ul chloroform and shake 2 minutes. Let stand 10 minutes.

(h) Centrifuge at 2000 rpm for 2 minutes. Inject 2 to 10 ul of chloroform taken from the bottom of tube after pre-wetting syringe with chloroform.

5.033F. Calculations for FPD Detection:

(a) Measure the peak heights for the levamisole and internal standard. Calculate the ratio of the levamisole standard to the internal standard.

The y axis is the $\log (\text{ratio} \times 10)$

The x axis is the $\log [(\text{Lev. ppm})^2 \times 1000]$

For example:

ppm Levamisol	ratio
0.05	0.36
0.10	1.06
0.20	2.88

Curve 1 is constructed from the following points:

X	y	x	y	
$\log [(0.05)^2 \times 1000]$	$\log [(0.36 \times 10)]$	0.04	0.56	$r = 0.997$
$\log [(0.10)^2 \times 1000]$	$\log [(1.06 \times 10)]$	1.00	1.03	$m = 0.75$
$\log [(0.20)^2 \times 1000]$	$\log [(2.88 \times 10)]$	1.60	1.46	$b = 0.267$

$$\text{ppm Levamisol} = \sqrt{\frac{\text{antilog} \left[\frac{\log (\text{ratio} \times 10) - \text{intercept from std. curve}}{\text{slope of std. curve}} \right]}{1000}}$$

If a sample had a levamisole peak of 75 mm with an internal standard of 60 mm, the following calculation would be made:

$$\frac{75 \text{ mm}}{60 \text{ mm}} = 1.25 \quad \text{Because } \begin{array}{l} y = \log (\text{ratio} \times 10) \\ y = \log 12.5 = 1.09691 \end{array}$$

$$y = mx + b$$

$$1.09691 = (0.75)(x) + 0.267$$

$$\frac{1.09691 - 0.267}{0.75} = x = 1.1065$$

$$\text{Because } x = \log [(\text{ppm Levamisole})^2 \times 1000]$$

$$1.1065 = \log [(\text{ppm Levamisole})^2 \times 1000]$$

$$\text{antilog } 1.1065 = (\text{ppm Levamisole})^2 \times 1000$$

$$12.77 = (\text{ppm Levamisole})^2 \times 1000$$

$$\sqrt{\frac{12.77}{1000}} = \text{Levamisole ppm}$$

$$0.11 = \text{ppm Levamisole}$$

(b) A more simplified calculation follows:

The y axis is the ratio of Levamisole vs. Internal Standard

The x axis is the (ppm levamisole standard)²

Curve II is constructed from the following points:

ppm Levamisole ²	ratio	
0.0025	0.36	r = 0.9966
0.01	1.06	m = 65.3333
0.04	2.88	b = 0.29

If a sample had a Levamisole peak of 75 mm with an internal standard of 60mm, the following calculations would be made:

$$\text{ppm Levamisole} = \sqrt{\frac{(\text{ratio}) - (\text{intercept})}{\text{slope}}}$$

$$\frac{75 \text{ mm}}{60 \text{ mm}} = 1.25 \quad \text{Because } \begin{matrix} y = \text{ratio} \\ y = 1.25 \end{matrix}$$

$$y = mx + b$$

$$1.25 = (65.333) (x) + 0.29$$

$$x = \frac{1.25 - 0.29}{65.3333} = 0.0147$$

Because $x = (\text{ppm Levamisole})^2$

$$\text{ppm Levamisole} = \sqrt{x}$$

$$= \sqrt{0.0147}$$

$$= 0.12 \text{ ppm Levamisole}$$

This calculation is easier to handle than the previous, although not as exact. However, for a screening procedure, it estimates the amount present. Using the later calculation is recommended. Any samples calculated to be 0.085 ppm or higher must be confirmed.

Notes:

1. The sodium sulfate addition has two functions: to partially dry the organic solvent (ethyl acetate) and to act as a physical barrier between the tissue and the 50 percent KOH. If the base is added directly on top of the liver, it will tend to coalesce the tissue and prevent an adequate mix.

2. Chloroform extract of levamisole is stable at 5°C for approximately 3 to 5 days.
3. For off-scale peaks, samples can be diluted by adding an appropriate amount of chloroform to the sample tube and shaking approximately 30 seconds.

5.034 Determination and Confirmation of Albendazole Residues in Animal Tissue

5.034A. Theory

This method determines residue concentrations in liver by HPLC, using a fluorescence detector with a lower limit of detection of 125 ppb. Residue findings may be confirmed by GC/MS.

Homogenized cattle liver (2.5 g) is fortified with an internal standard [SK&F 101437-C, 5- (Butylsulfonyl)-1H-benzimidazol-2-amine], hydrolyzed in 6N hydrochloric acid, adjusted to $\text{pH} \geq 8.0$ with sodium carbonate and extracted with ethyl acetate. The albendazole marker and internal standard are back-extracted into 1N hydrochloric acid and the ethyl acetate removed by aspiration. The aqueous phase is adjusted to $\text{pH} > 8.0$ with sodium carbonate, and then extracted with toluene which is removed by aspiration. The aqueous phase is applied to a SEP-PAK[®]C₁₈ cartridge where the compounds of interest are retained and eluted with ethyl acetate. The ethyl acetate extract is then concentrated to dryness. The residue is reconstituted in mobile phase, filtered and quantification performed by HPLC using fluorescence detection.

5.034B. Apparatus

- (a) Glass graduated centrifuge tube. 50 ml ground glass stoppered, Kimble 45176-50, or equivalent.
- (b) Glass graduated centrifuge tube, 13 ml ground glass stoppered, Kimble 45176-13, or equivalent.
- (c) Oven, Fisher Isotemp Model 176 equipped with thermometer, Fisher Scientific Co., or equivalent.
- (d) Mechanical shaker, Eberbach flatbed, A. H. Thomas 8287-E30, or equivalent.
- (e) Centrifuge, Sorvall Model GLC-1, equipped with rotors for 50 ml and 15 ml tubes.
- (f) N-Evap[®], Model 112, Organomation Assoc., Inc., or equivalent.
- (g) Commercial Blender, Waring Model 31BL91, or equivalent.
- (h) Pipetter, Becton Dickinson Labware (BDL) Model 6606, or equivalent.
- (i) pH meter, Radiometer Model 22, or equivalent.
- (j) SEP-PAK[®]C₁₈ cartridges, Waters Assoc. (Part #51910).
- (k) Vortex-Genie Mixer, Model K-550-G, Fisher Scientific Co.
- (l) Filter assembly, MF-1 Centrifugal Microfilter, Bioanalytical Systems Inc.
- (m) Filters, 0.2µm RC58 (Regenerated cellulose), Bioanalytical Systems Inc.
- (n) Balance, top loading Sartorius model 1219MP, or equivalent.
- (o) Dry ice or ice.
- (p) Pipet, 10 ml glass disposable, A. H. Thomas (7536-H85), Corning #7077, or equivalent.
- (q) Pipet, Pasteur disposable, A. H. Thomas 7760-A10, 7760-A26, or equivalent.
- (r) Pipet-Aid[®], Drummond Scientific Co.
- (s) Syringe, 10 mL glass luer tip hypodermic, A. H. Thomas, 8932-D50, or equivalent.

- (t) Electrobalance, Cahn, Model G-2, or equivalent.
- (u) HPLC column, u-BONDAPAK™ C₁₈, 30 cm × 3.9 mm I.D., Waters Associates (P/N 27324).
- (v) HPLC Microprocessor/controller, Model 420. Altex Scientific Inc., or equivalent.
- (w) Guard column, CO:PELL ODS, 2mm ID × 5cm, Whatman, Inc., or LiChrosorb 10 um Brownlee Labs MPLC cartridge, or equivalent.
- (x) HPLC Pump, Model 100, Altex Scientific Inc., or equivalent.
- (y) HPLC Injection valve, Model 7120 equipped with 20 ul loop, Rheodyne, Inc., or equivalent, or automatic sampler.
- (z) Recording Intergrator, Model 5880, Hewlett Packard, or equivalent.
- (aa) HPLC Fluorescence Spectrophotometer, Model 204A, Perkin-Elmer Corp., or equivalent dual monochromator instrument.
- (bb) Syringe Model LC-210, Pressure-Lok®, equipped with Rheodyne needle, Precision Sampling Corp., or equivalent.
- (cc) Xenon Power Supply, Model 150, Perkin-Elmer Corp.
- (dd) Vacuum filter, 47mm glass, Millipore Corp.
- (ee) Filter, Nylon-66 membrane 0.45um, 47mm diameter, Rainin Instrument Co., or equivalent.
- (ff) Syringe, 10 ul glass, Hamilton, Model 1801, or equivalent.
- (gg) Syringe, 50 ul glass Pressure-Lok®, Precision Sampling, Model C-160, or equivalent.
- (hh) Fused silica capillary column, DB-5 liquid phase, 0.25um film thickness, 30m × 0.25mm i.d., J & W Scientific, Inc., or equivalent.
- (ii) Gas Chromatograph/Mass Spectrometer, Finnegan Corporation, Model 4500 equipped with Model 9610 gas chromatograph, or equivalent.

5.034C. Reagents

- (a) SK&F 5-(Propylsulfonyl)-1*H* benzimidazol-2-amine, analytical standard, Smithkline Animal Health Products, 1600 Paoli Pike, West Chester, PA 19380.
- (b) SK&F 101437, 5-(Butylsulfonyl)-1*H* benzimidazol-2-amine, analytical standard, Smithkline Animal health Products.
- (c) Hydrochloric acid, concentrated, J. T. Baker Chemical Co., #1-9535, or equivalent.
- (d) Dimethylsulfoxide (DMSO), distilled in glass, Burdick & Jackson Laboratories, or equivalent.
- (e) Ethyl Acétate, distilled in glass, Burdick & Jackson Laboratories, or equivalent.
- (f) Methanol, distilled in glass, Burdick & Jackson Laboratories, or equivalent.
- (g) Toluene, distilled in glass, Burdick & Jackson Laboratories, or equivalent.

- (h) Acetonitrile, distilled in glass, Burdick & Jackson Laboratories, or equivalent.
- (i) Diethanolamine, Fisher Scientific Co., D-45, or equivalent.
- (j) Sodium Carbonate, anhydrous powder, Mallinckrodt, Inc. #7521, or equivalent.
- (k) Water, via Milli-Q/Milli-RO system, Millipore Corp., or equivalent.
- (l) Potassium Phosphate Monobasic, Fisher Scientific Co. P-285, or equivalent.
- (m) Potassium Phosphate Dibasic, anhydrous, Mallinckrodt, Inc., #7092, or equivalent.
- (n) Nitrogen, Prepurified grade, M. G. Burdett Gas Products Co., or equivalent.
- (o) N-Methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), Regis Chemical Co.

5.034D. Standard Solutions — Determinative Assay

(a) Stock Solutions (Extraction):

(1) Into a 100 ml volumetric flask, weigh 10.0 mg of SK&F 81038 analytical standard. Dissolve and take to mark with DMSO (100 ug/ml).

(2) Into a 100 ml volumetric flask, weigh 10.0 mg of SK&F 101437 analytical standard. Dissolve and take to mark with DMSO (100 ug/ml).

(b) Working Standards (Extraction):

(1) Pipet 2.0 ml of stock solution (a)(1) and 8.0 mL of DMSO into a 13 mL glass stoppered centrifuge tube and vortex mix, to produce a 20 ug/ml solution (b)(1) (50 ul fortified to 2.5 g of liver is equivalent to 400 ppb).

(2) Pipet 5.0 ml of working standard (b)(1) and 5.0 mL of DMSO into a 13 mL glass stoppered centrifuge tube and vortex mix, to produce a 10 ug/ml solution (b)(2) (50 ul fortified to 2.5 g of liver is equivalent to 200 ppb).

(3) Pipet 5.0 ml of working standard (b)(2) and 5.0 ml of DMSO into 13 mL glass stoppered centrifuge tube and vortex mix, to produce a 5 ug/ml solution (b)(3) (50 ul fortified to 2.5 g of liver is equivalent to 100 ppb).

(4) Pipet 1.0 ml of stock solution (a)(2) and 9.0 ml of DMSO into a 13 ml glass stoppered centrifuge tube and vortex mix, to produce a 10 ug/ml solution (b)(4) (50 ul fortified to 2.5 g of liver is equivalent to 200 ppb).

All standard solutions should be stored in a refrigerator. Standard solutions are stable in DMSO for a minimum of six months. Because DMSO solidifies under these conditions it is necessary to re-liquify the standard solutions by placing the tubes in a beaker of lukewarm water and vortex mix the solutions prior to use.

5.034E. Standard Solutions - GC/MS Confirmation

(a) Prepare standard solutions (GC/MS) as described in 5.034D. (a) and (b) above, using methanol rather than DMSO

(b) Into separate 13 ml glass centrifuge tubes, prepare standards equivalent to 100, 200, and 400 ppb based upon a 2.5 g tissue sample weight as follows:

Pipet 50 ul of working standard (b)(4) into each of three 13 ml glass tubes. Pipet 50 ul of each of the working standards (b)(1), (b)(2), and (b)(3) into a separate 13 ml tube containing working standard (b)(4).

5.034F. Determination

(a) Representative sections of cattle liver are thoroughly homogenized in a commercial Waring Blender until no visible pieces remain (approximately 3-5 minutes/100 g). Homogenates should be kept frozen at all times until analyzed.

(b) Into the bottom of a 50 ml glass centrifuge tube (unstoppered), supported in a beaker tared on a balance, weigh 2.5 ± 0.05 g of homogenized liver using a wide tip disposable pipet. Prepare four blank tissue samples, one for control and three for fortification. Add 100 μ l of pure DMSO to the control. Add 50 μ l of DMSO to the tissues suspected of containing albendazole residue. Add 50 μ l of working standard solution (b)(4) to each of the remaining three blank tissue samples and the tissue samples suspected of containing albendazole residue. Add 50 μ l of working standard solution (b)(1), (b)(2), (b)(3), to separate blank tissue samples from the set of three already fortified with working standard solution (b)(4). (Note: Fortification solutions should be added to the 50 ml tube just above the homogenate to insure solution when the HCl is added in the next step).

(c) Add 2.5 ml of 6N HCl to each sample prepared in step (b), stopper each tube, place the sample tubes (covered with another tube rack to prevent stoppers from popping) in an oven preset to $110 \pm 4^\circ\text{C}$ (at elevations ≤ 1000 ft above sea level).*

(d) After 1 hour remove the samples from the $110 \pm 4^\circ\text{C}$ oven and allow the tubes to cool to room temperature over dry ice or ice for approximately 5 minutes.

(e) Add 2.0 ml of water (Milli-Q) to each sample. While vortex mixing the sample, slowly add enough (approximately 1.23 g) of sodium carbonate (approximately 4-5 minutes to avoid foaming and/or caking) to achieve a pH 8-9.5 when monitored with a pH meter. (Place the clean pH electrode directly into the sample. After recording the pH, dab any drop of solution from the electrode on the inside of the tube. Completely remove the electrode from the tube, rinse it with water, and dry it with a Kimwipe before monitoring the next sample.) Note: Samples at or near room temperature do not foam as much as colder samples. Therefore, the Na_2CO_3 can be added faster.

(f) Add 15 ml of ethyl acetate to each sample and stopper the tubes. Shake the samples, supported in a rack in a horizontal position for 5 minutes. Remove the stoppers and centrifuge the samples at approximately 400 g for 5 minutes.

(g) Transfer as much as possible of the ethyl acetate extracts to separate 50 ml glass centrifuge tubes using a disposable pipet *taking care not to transfer any of the aqueous phase* (use of a Pipet-Aid® in this step is beneficial).

(h) Re-extract each sample a second time by repeating steps (f) and (g) and pooling the appropriate extracts in the respective 50 ml glass centrifuge tubes.

(i) Add 4.0 mL of 1N HCl to each ethyl acetate pooled extract and stopper the tubes. Shake the samples supported in a rack in a horizontal position for 5 minutes. Remove the stoppers and centrifuge the samples at approximately 400 g for 5 minutes.

(j) Remove as much as possible of the ethyl acetate phase by aspiration, *taking care not to remove any of the aqueous phase*. Evaporate the remaining ethyl acetate (< 1 ml) from the aqueous phase in the N-Evap® waterbath at $35 \pm 5^\circ\text{C}$. (If necessary the extraction can be stopped at this point overnight.)

(k) While vortex mixing each sample, add enough (approximately 0.33 g) of sodium carbonate slowly (approximately 1-2 minutes to avoid foaming and/or caking). Each sample should be at pH 8-9.5 when monitored with a pH meter.

(l) Add 20 ml of toluene to each sample and stopper tubes. Shake the samples, supported in a rack in a horizontal position, for 5 minutes. Remove the stoppers and centrifuge the samples at approximately 400 g for 5 minutes.

* At elevations > 5000 ft, hydrolysis for 30 minutes in a 15 psi pressure vessel is required to adjust for atmospheric pressure. Timing for hydrolysis should begin when the samples are placed into the pressure vessel with the water already boiling. It is necessary to have the water boiling to reach water pressure as quickly as possible (≤ 4 minutes). The hydrolysis time is critical and must be timed accurately.

(m) Remove as much as possible of the toluene phase by aspiration *taking care not to remove any of the aqueous phase*. Evaporate the remaining toluene (< 1 ml) from above the aqueous phase in the N-EVAP[®] waterbath at $35 \pm 5^{\circ}\text{C}$. (If necessary the extraction can be stopped at this point overnight).

(n) Attach a SEP-PAK[®]C₁₈ cartridge to the luer tip of a 10 ml glass syringe secured to a ring stand with a clamp for each sample (cylinder barrel removed).

(o) Prewash each cartridge with 2 ml of methanol followed by 2 ml of 0.2M potassium phosphate buffer, pH 8.0. Each reagent is transported through the cartridge by means of gentle nitrogen pressure applied through a rubber stopper fitted into the top of the syringe after addition of the respective reagent. The nitrogen pressure is removed when nitrogen begins to exit the cartridge. Flow rate approx. 1-2 ml/min. Discard eluates.

(p) Transfer the aqueous phase from each sample from step (m) to a separate prewashed syringe cartridge unit. Elute this phase from the cartridge using nitrogen as in step (o) then transfer a 1 ml water (Milli-Q) wash of each sample tube (vortex-mixed) to the respective syringe-cartridge unit in a similar manner using the respective pasteur pipet. Discard all eluates.

(q) Add an additional 1 ml water (Milli-Q) wash of each sample tube (vortex mixed) to the respective syringe-cartridge unit and elute using nitrogen as in step (o). Discard eluates.

(r) Add 2 ml of toluene to each syringe-cartridge unit and elute using nitrogen as in step (o). Discard eluates.

(s) Place a 13 ml glass centrifuge tube beneath each syringe cartridge unit. Add 2 ml of ethyl acetate to each unit and elute using nitrogen as in step (o). This fraction contains the compound of interest.

(t) Add about 0.5 ml of methanol to each eluate from step (s) and vortex mix to obtain a homogeneous solution.

(u) Concentrate the eluates from step (t) to dryness under dry nitrogen in an N-EVAP[®] waterbath at $35 \pm 5^{\circ}\text{C}$.

(v) After reaching dryness add 0.5 ml of the water/methanol (70/30) solution and vortex mix to reconstitute the residue.

(w) After > 30 minutes apply each of the reconstituted samples to a separate Bioanalytical System filter unit fitted with an 0.2 μm regenerated cellulose filter. Centrifuge each filter unit containing a sample extract at approximately 400 g for 5-10 minutes.

(x) Transfer the filtrates to separate 13 ml glass stoppered centrifuge tubes and adjust the volume of each filtrate to 0.5 ml with water/methanol (70/30). Use of a silanized tube is helpful to concentrate the extract residue to the very bottom of the tube. After vortex mixing remove 0.1 ml for HPLC analysis (Determinative Procedure) and concentrate the remaining 0.4 ml of the liver extract samples along with the standard samples prepared as outlined in 5.034E., above, to dryness in an N-EVAP[®] waterbath at $35 \pm 5^{\circ}\text{C}$. If necessary reconstitute the residue in 50 μl of methanol and concentrate the samples to dryness such that the majority of residue is at or below the 100 μl mark on the tube. The samples should be concentrated using dry air. Nitrogen may be used in place of air but the samples should be flushed with air momentarily if immediately proceeding to the next step.

(y) Add 50 μl of MTBSTFA reagent to each concentrated sample. stopper tubes and vortex mix the samples. Place the stoppered sample tubes (covered with another tube rack to prevent stoppers from popping) in an oven preset to $100 \pm 4^{\circ}\text{C}$ to assure complete derivatization. Remove the samples from the oven approximately every half hour for vortex mixing then immediately return the tubes to the oven. After a total derivatization time of 2 hours remove the samples from the oven for GC/MS analysis. The samples may remain in the oven longer. Two hours derivatization is a minimum. If a large number of samples are prepared for analysis, it is recommended that each sample remain in the oven until analysis begins.

Ideally the derivatized samples should be analyzed the day of derivatization. If an instrument malfunction prevails such that the samples cannot be analyzed on the day of derivatization, store the samples in a freezer, tightly stoppered and sealed with parafilm. Reheat the samples the following day for 1 hour at $100 \pm 4^{\circ}\text{C}$ prior to analysis. It is strongly suggested, however, to analyze the samples on the initial day of derivatization.

Notes:

1. If frozen cattle liver is used, it must be kept frozen until assay. Liver homogenate stored in small vials is thawed under lukewarm water prior to each assay. Brief thawing of either the frozen liver or frozen liver homogenate is acceptable, if re-frozen as soon as possible.

2. The Pipet-Aid[®] manufactured by Drummond Scientific Inc. is a convenient time-saving device for transferring or aspirating solutions.

3. A ≥ 75 percent recovery is achieved by taking care to transfer as much as possible of the extraction solvents (steps 5.034E (f), (g), and (h)). In cases where the solvent is discarded and the aqueous portion containing the compounds of interest is retained, it is imperative to avoid removing any of the aqueous layer (steps (j) and (k)). Transferring of the 4 ml aqueous phase in step 16 should be done quantitatively using a pasteur pipet. The 1 ml washes of the sample tube are transferred to the SEP-PAK[®]C₁₈ cartridge using the same pasteur pipet. When transferring solutions, separate disposable and pasteur pipets are used for each sample to avoid contamination.

4. The sample extracts contained in water/methanol (70/30) are stable for at least a month and if reanalyzed provide data similar to the previous HPLC analysis.

5. The sample extracts concentrated to dryness for derivatization and subsequent GC/MS analysis are stable for at least a month when stored in a refrigerator.

Verification of Control Cattle Liver

To determine that a cattle liver can be used as control proceed as follows: Prepare four 2.5 g liver homogenate samples. Fortify two aliquots with 100 μ l of DMSO and the other two aliquots with 50 μ l of DMSO and 50 μ l of the internal standard solution (b)(4) (200ppb). The samples are processed through the extraction method and analyzed using the HPLC procedure. The non-fortified control samples (DMSO only) should produce either no or only a negligible response at the retention time corresponding to the internal standard as compared to the response observed for a known control sample fortified at 200 ppb. Providing this background is negligible, calculate the background at the retention time corresponding to the albendazole marker in the control samples fortified with internal standard. If a response is observed, calculate the peak area (height) ratio. If either no response or peak area (height) ratio less than 5 percent of that calculated for a known control fortified at 200 ppb is determined, the cattle liver is considered a control.

5.034G. High Performance Liquid Chromatographic Analysis

Column: 30 cm x 3.9 mm I.D. μ BONDAPAK[®] C₁₈

Flow Rate: 1.8 ml/min

Mobile Phase: 68% 0.02M KH₂PO₄;0.01M diethanolamine (200 ml 0.1M KH₂PO₄ + 1.05g diethanolamine taken to 1 liter with Milli-Q water)
20% methanol
12% acetonitrile

This solution is prepared daily, filtered through a 0.45 μ m Nylon-66 filter and degassed under vacuum prior to use.

Fluorescence Detector:

Initially set the excitation and emission wavelengths at 300 nm and 320 nm, respectively, with a slit width of ≤ 10 nm for both. Inject an appropriate volume of sample (20 μ l) and observe the intensity of the response for both SK&F 81038 and SK&F 101437 with a reasonable baseline sensitivity setting. If additional sensitivity is required proceed as follows:

While observing the detector absorption response, adjust the excitation wavelength to ≤ 5 nm to reduce the zero suppression. Reinject the same sample as above and determine if the signal responses for the compounds of interest

increase. Continue this procedure of optimizing both the excitation and emission wavelengths to obtain an adequate signal to noise ratio for the analysis ($s/n \geq 25/1$)

Temperature:

Column, mobile phase, and detector temperature = ambient ($70 \pm 2^\circ\text{F}$)

Approximate Retention time: Albendazole Marker (SK&F 81038
approximately 4.3 minutes)

Internal Standard (SK&F 101437
approximately 7.0 minutes)

5.034H. Data Computation

If an integrator is unavailable, peak height measurements and peak height ratio data can be substituted for peak area and peak area ratio data in the following discussion.

Divide the peak area of the albendazole marker SK&F 81038 by the peak area of the internal standard SK&F 101437 from the HPLC data for each sample extract, to obtain peak area ratios.

$$\text{peak area ratio} = \frac{\text{Area of albendazole marker peak (SK\&F 81038)}}{\text{Area of internal standard peak (SK\&F 101437)}}$$

Construct an analytical curve by plotting, on linear axes, the peak area ratio versus the respective ppb value for each fortified control sample. Using the regression equation for the analytical curve, directly calculate the ppb level of albendazole marker in the unknown samples from their peak area ratios using the following equation:

$$x = \frac{y - b}{m}$$

where, x = ppb concentration in suspect samples

y = peak area ratio of suspect sample extract

b = intercept from regression equation

m = slope from regression equation

5.034I. Gas Chromatograph/Mass Spectrometer Analysis

(a) Gas Chromatograph Conditions:

Column: Fused Silica Capillary, 30 m x 0.25 mm i.d., DB-5 (0.25 μm film thickness)

Injection Port Temperature = 260°C

Column Oven Temperature Program:

Initial Temperature = 200°C (hold 1 minute)

Final Temperature = 300°C (hold for duration of analysis)

Temperature Program Rate = $20^\circ\text{C}/\text{minute}$

Injection Mode: Grob Splitless, split vent timing 0.6-1 minute vent flow 100 ml/minute

Carrier Gas: Helium, 30-40 psi

Linear Velocity = 60-70 cm/second

Approximate Retention Times (t_R):

Marker Residue (SK&F 81038), approximately 8 minutes

Internal Standard (SF&K 101437), approximately 9 minutes

Injection Volume: 1.0-3.0 μ l.

(b) Mass Spectrometer Conditions:

Temperatures:

Source = 140°C (Model 4500), 225°C (Model 4000 dial setting 30)

Separator = 275 \pm 5°C

Transfer Line = 275 \pm 5°C

Manifold = 120°C (Model 4500), 100 \pm 5°C (Model 4000)

Multiplier: Gain = 5×10^4 to 1×10^5

Voltage = 2000 to 2400 eV

Election Energy = 70 eV

Calibration Standard: FC-43 (Perfluorotributylamine)

Emission Current = -0.30

Preamplifier Sensitivity = 10^{-7} to 10^{-8}

(c) Gas Chromatograph/Mass Spectrometer Procedure:

After initial instrument calibration to ≥ 500 amu, perform partial scan analysis (mass range 45-500 amu) with an appropriate amount (3.0 μ l) of a standard sample containing 10 μ g of SK&F 81038 and 10 μ g of SK&F 101437 as the t-butyldimethylsilyl (t-BDMS) derivatives in 50 μ l of derivatizing reagent. Determine the centroid mass assignments of the ions 189, 354, 410, 467 for SK&F 81038-t-BDMS and 189, 368, 424, and 481 for SK & F 101437-t-BDMS by averaging the observed centroid masses over approximately five scans of the respective chromatographic peaks or by using a procedure applicable to the particular data system employed. The instrument should be calibrated to obtain a minimum relative intensity of 10 percent for each ion monitored. Set the multiple ion detection mode to monitor these seven ions. Select a beginning and end mass range for each ion and scan time appropriate for the particular instrument employed.

Proceed as follows for the Multiple Ion Detection (MID) Analysis:

1. Perform three analyses of the derivatized standard samples from 5.034E. (equivalent to 100, 200, and 400 ppb) with an appropriate injection volume (approximately 350 μ l). From these analyses determine the relative ion intensity reproducibility for the instrument employed.

2. Analyze an aliquot (approximately 3.0 μ l) of the derivatization reagent (MTBSTFA) to insure there is no ghosting interference with the monitored ions at the retention times corresponding to the components of interest.

3. Analyze an appropriate amount (approximately 3.0 ul) of the derivatized control and fortified control (100, 200, and 400 ppb) cattle liver extracts, respectively. Determine the relative ion intensity reproducibility from these samples. The relative ion intensity for a particular ion should not vary by more than ± 10 percent.

4. Repeat step 5.034I (c) 2, above.

5. Providing the relative ion intensity is reproducible to within ± 10 percent and no interference is apparent due to ghosting, proceed to analyze the derivatized cattle liver extracts suspected of containing albendazole residues. Alternate injections of each suspect sample with injections of the derivatization reagent (step 2) to insure that there is no interference due to ghosting between analyses of the suspect samples.

6. Record the relative intensity data for ions 189, 354, 410, and 467 for SK&F 81038-t-BDMS and 189, 368, 424, and 481 for SK&F 101437-t-BDMS from each analysis. Confirmation of the albendazole marker residue in the suspect cattle liver samples consists of: 1) identifying a response at the retention time corresponding to the albendazole marker t-BDMS derivative, 2) this response containing the four characteristic mass ions ($m/e = 189, 354, 410$, and 467), and 3) the relative ion intensity of each ion reproducible to within ± 10 percent of that obtained for these ions from the derivatized control cattle liver extracts fortified with the albendazole marker chemical.

5.034J. Supplemental Mass Spectral Information

A summary of the most reasonable exact mass assignments and elemental compositions of the ions of interest for the electron impact mass spectrum of the albendazole marker t-BDMS derivative is listed in Table 1. These data were obtained with a high resolution VG Model 7070 magnetic sector instrument. The m/e 354 ion was saturated deliberately to enhance several of the weaker ions. These data are presented merely as supplemental information to support the rational for choosing the ions to monitor.

TABLE 1
EXACT MASSES AND ELEMENTAL COMPOSITIONS OF IONS
FOR ALBENDAZOLE MARKER t-BDMS DERIVATIVE

Nominal Mass	Exact Mass	Elemental Composition	Error (ppm)
130	130.0507	$C_7H_{12}NSi_2$	1.6
189	189.0721	$C_9H_{11}N_3Si$	4.2
236 ^a	—	—	—
246	246.0881	$C_{11}H_{16}N_3Si_2$	2.0
296	296.0886	$C_{12}H_{18}N_3O_2SSi$	-0.2
303	303.1585	$C_{15}H_{25}N_3Si_2$	0.6
340	340.0969	$C_{13}H_{22}N_3O_2SSi_2$	1.4
354	354.1126	$C_{11}H_{21}N_3O_2SSi_2$	7.4
396	396.1596	$C_{17}H_{30}N_3O_2SSi_2$	-7.1
410	410.1752	$C_{18}H_{32}N_3O_2SSi_2$	-4.2
452	452.2221	$C_{21}H_{38}N_3O_2SSi_2$	-10.1
467	467.2455	$C_{22}H_{41}N_3O_2SSi_2$	-11.5

^a No reasonable compositions

5.035 DETERMINATION AND CONFIRMATION OF IVERMECTIN RESIDUE IN ANIMAL TISSUE

5.035A. Theory

This assay procedure for ivermectin in animal tissue was developed at Merck Sharp and Dhome Research laboratories, Rahway, New Jersey.

Avermectins, which are isolated from the mycelia of streptomyces avermitilis, are new, broad spectrum family of compounds which are potent agents at very low dosage levels. (Egerton et. al. 1979). Ivermectin, itself, is a mixture of two homologues, not less than 80 percent 22,23-dihydroavermectin B1a or H2B1a and not more than 20 percent 22,23-dihydroavermectin B1b or H2B1b. Figure 1 shows the basic structure of these compounds.

Radio-metabolism studies conducted at Merck laboratories have demonstrated that the H2B1b is metabolized more rapidly than H2B1a. In addition, these studies concluded that H2B1a is not rapidly metabolized and, hence, is the major residue found at the proposed withdrawal times. Thus, 22,23-dihydroavermectin B1a appears to qualify as the correct marker substance, with liver tissue the target tissue.

Tolan and co-workers (1980) developed a HPLC method using fluorescence detection for avermectins in plasma. Modification of the Tolán method to apply it to tissue resulted in a procedure which is shorter and more reproducible. The method determines H2B1a rapidly at a lower limit of reliable measurement of 10 ppb or less. The procedure involves conversion of H2B1a to the fluorescent derivative (Figure 1) which is readily chromatographed and sensitively and selectively detected. The method developed has been applied to liver samples as well as kidney, muscle, fat, and plasma. (Tway et. al. 1981). Tissue from cattle, sheep, horses, and swine have been successfully assayed.

The tissue is homogenized with acetone-water and the dihydro B1a is extracted with isooctane with the tissue present. Following removal of the isooctane, solvent-solvent distributions into acetonitrile out of hexane and into hexane out of acetonitrile-water are performed. Solvent is removed and fluorescence produced by heating at 95°C in imidazole reagent. After adding chloroform, the reaction mixture is washed through a silica gel Sep-Pak, the solvent removed, and LC analysis on Zorbax C-18 with fluorescent detection is performed.

The procedure is not intrinsically dangerous. Nevertheless, because ivermectin is a weak teratogen, analysts should wear rubber gloves while performing the method. Solvent transfers are best performed in a fume hood and homogenizations should be performed in an ice water bath.

5.035B. Apparatus

- (a) Balance - analytical, capable of weighing 1 mg accurately
- (b) Balance - capable of weighing 5 g accurately
- (c) Water bath - 40-80°C
- (d) Oil bath - 95-100°C
- (e) Centrifuge - IEC Model HN-S-II, with six place rotor and 15 ml and 50 ml carriers, or equivalent
- (f) Centrifuge tubes - 15 ml and 50 ml, glass with stoppers
- (g) Centrifuge tubes - 50 ml polypropylene (Corning #25331, or equivalent).
- (h) Centrifuge tubes - 15 ml, silylated approximately every 2 months. (Used only for derivitization reaction.) Pick tubes that the stoppers fit tightly. Fill each tube with Sylon-CT to the top. Let stand 20 minutes. Rinse with toluene then methanol. Fill with methanol and let stand 20 minutes. Rinse with acetone and dry. All glassware should be completely free of all acidic and alkaline residues. These tubes should be cleaned by hand, first by soaking in methylene chloride immediately after use and then in detergent for at least several hours followed by a thorough hot water rinse, distilled water rinse, and acetone rinse before drying. Variations of this washing technique has caused poor results for some analysts.

IVERMECTIN STRUCTURES.

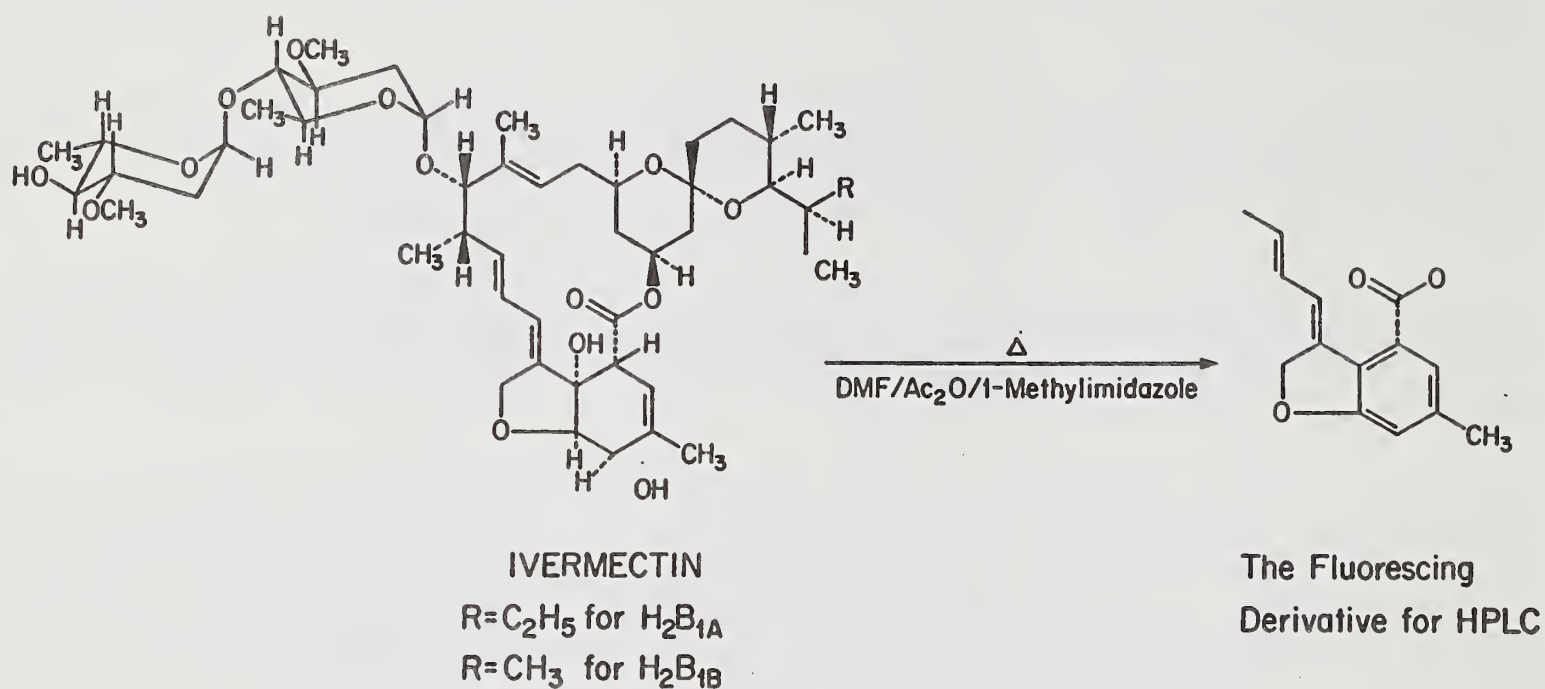


Figure 1

- (i) Dispensing pipetors - 10, 15, and 20 ml
- (j) Gloves disposable PVC (Fisher Chemical Co. or equivalent)
- (k) Freezer - Capable of $-20^{\circ}C$
- (l) Graduate - 25 ml
- (m) Pipets - disposable Pasteur
- (n) Pipets - graduated 1.0 and 2.0 ml
- (o) Parafilm
- (p) Pipets - volumetric 0.5, 1, 2, 3, 4, and 5 ml
- (q) Shaker - Eberbach, J. T. Baker #8287-E30, or equivalent
- (r) Sep-Pak - Silica gel (Waters #51900)

(s) Sorvall Omnimixer - Model 17150 with 50 ml stainless steel cups, or equivalent

(t) Syringe - 50 ul, 250 ul and 5 ml

(u) Tape - Ace Scientific #13-2956 1/2 inch

(v) Ultrasonic bath - Sonogen Automatic Cleaner, or equivalent

(w) Vortex mixer

(y) Liquid chromatograph - Waters liquid chromatograph consisting of Model 710B WISP sampler, model 6000 pump, model 720 fluorescence detector, Waters System Controller, and Waters Data Module. At 5 μ m, 4.6 mm id C-18 guard column (Brownlee Labs Spheri-5-RP-18) obtained from Rainin Instrument Co.) is used before the analytical column. This guard column is replaced monthly unless the pressure reaches 2000 psi, in which case it is replaced at that time.

Conditions: 15 cm length x 4.6 mm ID Zorbax ODS-C-18 column.

Mobile phase - 5 percent water in methanol (v/v).

Column Temperature = 30°C.

Flow - 1.8 ml/minute (usual pressure 1000 psi, 500-2500 psi acceptable).

Excitation Lamp - F4T5BL

Excitation Filter - 360 nm

Emission Filter - 425 nm

Retention time - 14 minutes

5.035C. Reagents

(a) Acetic anhydride - Baker, reagent grade, or equivalent.

(b) Acetone - Mallinkrodt, nanograde, or equivalent.

(c) Acetonitrile - Fisher, HPLC grade or Mallinckrodt, nanograde, or equivalent.

(d) Chloroform - Burdick and Jackson distilled in glass, or equivalent.

(e) Dimethyl Formamide (DMF) - Baker, reagent grade, or equivalent.

(f) Ethyl Acetate - Burdick and Jackson, distilled in glass, or equivalent.

(g) Hexane - Burdick and Jackson, distilled in glass, or Mallinckrodt, nanograde, or equivalent.

(h) Methanol - Burdick and Jackson, distilled in glass or Mallinckrodt, nanograde, or equivalent.

(i) Methylene Chloride - any grade available.

(j) 1-Methylimidazole - 99 percent Aldrich Chemical Company, or equivalent.

(k) Nitrogen - the equivalent of Matheson extra dry compressed gas.

(l) Sodium Chloride - Baker, reagent grade, or equivalent.

(m) Sylon-CT - Supelco, Inc.

(n) 2, 2, 4-Trimethylpentane - (Isooctane), Mallinckrodt, nanograde, or equivalent.

(o) Water, double distilled - distilled, deionized water redistilled in an all-glass apparatus.

(p) Derivatizing Reagent - Mix 0.2 ml of 1-methylimidazole with 0.3 ml of acetic anhydride and 0.9 ml of DMF. Prepare just before use.

(q) Acetone/Water - 50 percent (v/v).

(r) Methanol/Water - Make 100 ml of doubly distilled and filtered water to 2 l with methanol. Deaerate by slowly bubbling nitrogen through for 10 minutes.

(s) Standard Solutions.

1. Solid H2B1a and H2B1b are not available in easily handled forms. In addition to long-term potential instability, they both readily gain and lose moisture with changing humidities. As a consequence solutions of standard in solvents like propylene glycol are a more satisfactory source. The concentrated propylene glycol solution is prepared by weight and its concentration verified by conventional LC techniques.

2. Standards for the LC verification must still be weighed with concomitant purity and moisture difficulties, but these difficulties can be overcome by experts in the handling of ivermectin. Also, the preparation of standard glycol solutions need be done only rarely. Again, the glycol solution is best protected from moisture and should be stored at -20°C and allowed to come to room temperature before opening to weigh a sample. Dilutions of standard are made in methanol.

3. Another problem involving dilute solutions of ivermectin involves stability. Such solutions are unstable to even trace amounts of acid, alkali, and detergents and also to air and light. Even in the absence of all of these, ivermectin is often lost on glassware and/or plasticware. Fortunately the difficulties involving air and light are largely avoided by using alcoholic solvents and those involving acids and bases handled by sufficient cleaning of all vessels used in the analysis. Other glassware losses are avoided by sticking closely to the described procedures. When stored at -20°C, the most dilute methanolic standard solution has been shown to be completely stable for at least 1 year.

4. For the most sensitive scale, accurate aliquots of 50, 100, 150, 200 μ l of a 500 ng/ml standard solution of dihydro B1a in methanol are added to silylated 15 ml centrifuge tubes and the tubes blown to dryness at 60°C with nitrogen purge. After reaction, Sep-Pak treatment, etc., these samples are redissolved in 1 ml of methanol to give 25, 50, 75, 100, and 125 ng/ml standard solution equivalents for use on the 0.2 scale. For samples expected to contain higher amounts of dihydro B1a, larger amounts of standard are used and less sensitive fluorescence scales are employed. Generally, however, even unknown samples in the ppm range can be diluted before the LC step, to fit the 25-125 ng/ml scale.

5. Starting with solutions of about 0.045 percent (w/w) dihydro B1a in propylene glycol, stock solutions of about 10 micrograms/ml can be prepared by accurately weighing about 1 gram of the solution into a 50 ml volumetric flask. Methanol is used to dilute to the mark, and the solution is thoroughly mixed. Storage of this stock solution and all dilutions of same should be in polypropylene tubes at -20°C.

6. Analysts attempting the ivermectin residue analysis for the first time, would be advised to run a standard curve first before attempting the complete method.

5.035D. Determination

All Tissues

(a) Weigh accurately 5.0 ± 0.1 gram of tissue into a 50 ml homogenizer cup.

Liver, Muscle, Kidney

(b) Add 15 ml of 50/50 acetone/water into the cup and homogenize for two minutes.

(c) Pour the sample carefully into a 50 ml centrifuge tube making sure that everything possible goes into the tube.

Wash the cup and blades off into the centrifuge tube with small portions of isooctane until a total of 15 ml of isooctane is used.

Fat Only

(b) Add 20 ml of acetone/water and 10 ml of isooctane to the cup and homogenize two minutes.

(c) Pour the sample into a 50 ml centrifuge tube. In general much care must be used not to lose sample. A spatula may be needed to help effect the transfer.

(d) Wash the cup and blades off into the centrifuge tube with small portions of isooctane until a total of 10 additional ml of isooctane is used. Add 1 gram of solid sodium chloride to the tube.

All Tissue

(e) Stopper the tube, shake well for one minute, and centrifuge for 10 minutes at 2000-2500 rpm.

(f) Transfer the upper isooctane layer to a second 50 ml centrifuge tube with a disposable pipet. Completely avoid the lower layer.

(g) Break up the plug by using a vortex mixer and/or shaking.

(h) Add 15 ml of fresh isooctane and repeat the extraction combining the isooctane layers.

(i) Blow the combined isooctane layers down to a small volume (or ca. 5 ml for fat) using 80°C water bath and nitrogen purge.

(j) Repeat the extractions with two more passes of 15 ml of isooctane adding it in each case to the same tube which had the previous two isooctane layers.

(k) Again blow down as far as possible using 80°C bath and nitrogen purge.

(l) Add 6 ml of methanol to all samples (except fat) and dissolve or resuspend completely with an ultrasonic bath and/or vortex mixer.

(m) Place all samples (except fat) in a refrigerator until thoroughly cooled (at this point the sample is best left overnight in the refrigerator).

Fat Only

(l) Either add 2 ml of hexane and 5 ml of acetonitrile to the still hot and molten fat or freeze overnight in a freezer at -20°C. For samples frozen overnight, remelt in an 80°C bath and add the hexane and acetonitrile the next day.

(m) Shake the still warm mixture thoroughly for ca. one minute and immediately centrifuge.

(n) Cool and use a disposable pipet to move the upper or acetonitrile layer into a fresh 15 ml centrifuge tube. For horse and swine samples, the last drop of acetonitrile normally forms a bubble which should be transferred as completely as possible by pulling at its center with the pipet. Sheep and cattle samples can be cooled briefly in ice water and the acetonitrile layer decanted off.

(o) Repeat the melting, extraction with 5 ml of acetonitrile, and transfer into the same 15 ml tube.

(p) Add 2 ml of hexane to the 15 ml tube, shake and then centrifuge, remove the upper hexane layer to waste by disposable pipet. Go to step w.

- (n) Centrifuge the cold 50 ml tube for 5 minutes and decant off the clear supernatant to a fresh 15 ml centrifuge tube.
- (o) Wash the residue in the 50 ml tube with 1 ml of methanol using a vortex mixer, centrifuge, and decant off in either step, recentrifuge the 15 ml tube and decant off into a new 15 ml tube to get a clear solution with which to continue the assay.)
- (p) For muscle only, do a further 2 ml hexane extraction of the sample and add to the combined methanol.
- (q) Using a 60°C bath and nitrogen purge, blow off all the methanol.
- (r) Add 3 ml of hexane to the tube previously containing the methanol and use ultrasound to remove all material from the walls of the tube.
- (s) Add 4 ml of acetonitrile and repeat the ultrasound treatment.
- (t) Shake thoroughly, centrifuge 5 minutes, and move the lower acetonitrile layer to a fresh 15 ml tube.
- (u) Re-extract the hexane layer with a second 4 ml of acetonitrile and combine acetonitrile layers.
- (v) Extract the combined acetonitrile layers with 1 ml of hexane, centrifuge to clear, and move the upper hexane layer to waste by disposable pipet.
- (w) Blow the acetonitrile solution to < 1 using a 60° water bath and nitrogen purge.
- (x) If the acetonitrile is < 1 ml, ml, make up to 1 ml with fresh acetonitrile.
- (y) Use ultrasound if necessary to get a homogeneous mix.
- (z) Add 4 ml of distilled water (2 ml for kidney samples) and 5 ml of hexane and shake ca. 1 minute, centrifuge for 5 minutes.
- (aa) Move the upper hexane layer to a fresh 15 ml tube using a disposable pipet. Avoid lower layer.
- (ab) Repeat with 5 ml and then 4 ml hexane extractions combining all three hexane extractions.
- (ac) Blow to dryness (or as near dryness as possible) using a 40°C water bath and nitrogen purge. (To get completely dry, more heat may be necessary at the end of the evaporation. The bath may go as high as 80°C at this point.)
- (ad) Take the residue up in exactly 1 ml of methanol using vortex or ultra sound to dissolve.
- (ae) Mix thoroughly and centrifuge for 5 minutes.
- (af) Pipet exactly 0.5 ml of the supernatant into the bottom of a silylated 15 ml centrifuge tube. Use a 0.5 ml volumetric pipet.
- (ag) Carefully blow off the methanol using a 60°C bath and nitrogen purge. Avoid spattering.
- (ah) Add 0.1 ml of freshly made acetic anhydride/methyl imidazole/DMF reagent to each sample tube and a series of standard tubes using a 1.0 ml measuring pipet.
- (ai) Stopper, vortex, and centrifuge for a few seconds.

(aj) Tape the stoppers in place and put all samples and standards in a well-stirred 95°C oil bath for one hour. The bottom of the tubes should be about one inch below the oil surface.

(ak) Remove the tubes, wash the oil off with acetone, and cool.

(al) Add about 1 ml of chloroform to each tube and vortex to mix.

(am) Wash a Sep-Pak cartridge with 3-4 ml of chloroform using a syringe to force the liquid through the cartridge.

(an) Add the sample by disposable pipet to the syringe and force it through the cartridge.

(ao) Wash the cartridge tube three times with chloroform using 1 ml each time adding the washes to the syringe and through the cartridge.

(ap) Elute the column with a further 8-9 ml of chloroform (3, 3, 2 or 3 ml).

(aq) Collect *all* the chloroform in a 15 ml centrifuge tube.

(ar) Evaporate the chloroform off with a 60°C water bath and nitrogen purge. Get the residue completely dry.

(as) Pipet 0.5 ml of methanol (or other suitable quantity) into the tube and use a vortex mixer and ultrasound to completely dissolve the residue.

(at) Centrifuge briefly and inject 50 ul of the supernatant of each sample and standard into the LC.

(au) Measure the peak height at the retention time of the derivative of dihydro-B1a as indicated by the standards.

(av) Plot a standard graph of peak height versus ng/ml of standard. The curve should be linear and should go through the origin.

(aw) Read ng/ml for each sample from the graph and calculate ppb as follows:

$$\text{ppb} = \text{ng/ml} \times \frac{V1}{V2} \times \frac{D}{G}$$

Where: V1 = ml sample solution at end of assay

V2 = ml sample taken for derivative

D = Dilution of sample at end of assay or 1 if no dilution is made.

G = Sample weight = 5 grams

5.035E. Suitable Stopping Places

(a) Suitable stopping places for storage up to 4 days.

1. In a refrigerator at 4°C

(i) Only in methanol after step 5.035D. (1).

2. In a freezer at -20°C.

(i) After step (k) for fat only.

- (ii) After step (ak) the derivatization.
- (iii) After step (as) dissolution from chloroform

If stopping places other than the above are needed, assay stability at these stopping places should first be demonstrated.

5.035F. Confirmation Assay

The basic method of determining ivermectin in tissue has been described in the *Journal of Agricultural and Food Chemistry* 29. 1059 (1981). This method determines the 2223, dihydroavermectin B1a (the major ingredient of ivermectin) as the marker substance. The method depends on formation of a fluorescent derivative by dehydration, followed by liquid chromatography of the derivative.

The subject of this method is the confirmation of residues determined by the original method, while still using the basic precepts of the original. Thus parts of the clean up tissue are converted to (1) the monosaccharide and (2) the aglycone with parts of the original remaining. All three portions are next converted to fluorescent derivatives and the liquid chromatography is run as in the earlier procedure. In the new procedure the isolation of the derivative is by solvent extraction instead of Sep-Pak treatment and, hence interferences would have to both Sep-Pak and solvent extract like ivermectin. The new compounds are formed by treatment with 1 percent sulfuric acid in isopropanol and methanol respectively. Thus, a potential interference would have to form two new compounds with exactly the same chromatographic properties as those of ivermectin in two very different mediums. All three peaks may be semi-quantitated.

5.035G. Reagents

- (a) Acetic anhydride-Baker, reagent grade, or equivalent
- (b) Acetonitrile-Fisher or Mallinckrodt, nanograde, or equivalent
- (c) Dimethyl Formamide (DMF)-Baker reagent
- (d) Hexane-Burdick and Jackson, distilled in glass or Mallinckrodt, nanograde, or equivalent
- (e) Isobutyl alcohol-Burdick and Jackson, distilled in glass, or equivalent
- (f) Isopropyl alcohol-Burdick and Jackson, distilled in glass, or equivalent
- (g) Methanol-Burdick and Jackson, distilled in glass or Mallinckrodt, nanograde, or equivalent
- (h) Methylene chloride-any grade available.
- (i) Methylene chloride-Burdick and Jackson, distilled in glass, or equivalent
- (j) 1-Methylimidazole-99 percent, Aldrich Chemical Co., or equivalent
- (k) Nitrogen Matheson extra dry compressed, or equivalent
- (l) Sylon-CT Supelco Co.
- (m) 2, 2, 4-Trimethylpentane (isooctane) Mallinckrodt, nanograde, or equivalent
- (n) Water double distilled, deionized
- (o) Derivatizing reagent-Mix 0.2 ml of 1-methylimidazole with 0.3 ml acetic anhydride and 0.9 ml DMF.
Make just before use

- (p) Acetone/water - 50 percent (v/v).
- (q) Methanol/water-Dilute 100 ml of distilled/deionized water to 2 liter with methanol. Deaerate by slowly bubbling nitrogen through for 10 minutes.
- (r) Methylene chloride/hexane/isobutyl alcohol-Fill a 500 ml stoppered graduate to the 200 ml mark with methylene chloride. Add hexane to the 500 ml mark and add 20 ml isobutyl alcohol.
- (s) 1 Percent isobutyl alcohol in hexane-Dilute 5 ml of isobutyl alcohol to 500 ml in graduated cylinder.
- (t) 1 Percent sulfuric acid in isopropanol-Pipet 0.5 ml of sulfuric acid carefully into about 40 ml of isopropanol in a 50 ml volumetric flask. Dilute to mark with isopropanol.
- (u) 1 Percent sulfuric acid in methanol-Pipet 0.5 ml sulfuric acid carefully into 40 ml of methanol in a 50 ml volumetric flask. Dilute to the mark with methanol.

5.035H. Apparatus

- (a) Balance - analytical, capable of weighing 1 mg accurately.
- (b) Balance - capable of weighing 5 gram accurately.
- (c) Bath - water, variable temperature 40-80°C.
- (d) Bath - oil, 95-100°C.
- (e) Centrifuge - IEC Model HN-S-II, or equivalent, with six place rotor for 15 ml and 50 ml tubes.
- (f) Centrifuge tubes - glass 15 ml and 50 ml with polypropylene stoppers.
- (g) Centrifuge tubes - 50 ml polypropylene, Corning #25331 (used for storing standards only), or equivalent.
- (h) Centrifuge tubes - 15 ml silylated (see determinative procedure for details).
- (i) Dispensing pipetors - 10, 15, and 20 ml.
- (j) Gloves - disposable PVC, Fisher Scientific, or equivalent.
- (k) Freezer - capable of -20°C.
- (l) Graduate cylinder - 25 ml and 500 ml.
- (m) Pipets - disposable.
- (n) Pipets - graduated, 1.0 ml and 2.0 ml.
- (o) Parafilm
- (p) Pipets - volumetric 0.5, 1, 2, 3, 4, and 5 ml.
- (q) Reciprocating shaker - Eberbach, J. T. Baker catalog #8287-E30, or equivalent.
- (r) Sep-Pak - Water Associates.
- (s) Sorvall Omnimixer model 17105, or equivalent.
- (t) Syringe - 50 ul, 250 ul and 5 ml.

- (u) Tape - #13-2956, 1/2 inch from Ace Scientific.
- (v) Ultrasonic bath - Sonogen Automatic Cleaner - Bronson Model 520, or equivalent.
- (w) Vortex mixer.
- (x) Liquid chromatographic apparatus as in 5.035B.(y).

5.035I. Standard Solutions

Dilute a standard ivermectin solution in methanol until each 2 ml of the solution contains five times the number of nanograms of ivermectin as the level in the meat in ppb.

5.035J. Confirmatory Procedure

- (a) Proceed with the assay described in the 5.035D. through step (ac).
- (b) Pipet exactly 2.0 ml of methanol into the 15 ml tube and use a combination of vortex mixing and ultrasound to completely dissolve the residue.
- (c) Pipet exactly 0.5 ml of the solution from step (b) into each of two fresh 15 ml tubes and blow completely dry with nitrogen in a 70°C bath. The dried samples generally look like a small drop of oil. All solvent must be removed: Store the remaining 1 ml of sample in a freezer (for later).
- (d) Add 0.1 ml of 1 percent sulfuric acid in methanol to one of the samples (the A sample) and 0.1 ml of 1 percent sulfuric acid in isopropanol to the other (the M sample).
- (e) Vortex the sample for 10 seconds, then ultrasound it, and vortex again.
- (f) Let stand at room temperature for 16-18 hours (overnight).
- (g) To all (A) samples and (A) standards at one time, add methanol to the 1 ml mark and quickly mix.
- (h) Add mixed solvent of 40 percent methylene chloride/hexane/isobutyl alcohol to the 7 ml mark.
- (i) Add distilled water to the 11 ml mark, shake for one minute, and centrifuge for 5 minutes.
- (j) Move the mixed solvent upper layer to a fresh silylated 15 ml tube using a disposable pipet. Move as much of the upper layer as possible but absolutely no lower phase should be moved.
- (k) Repeat the extraction a second time with 6 ml of the mixed solvent. Combine extracts in the silylated tube.
- (l) Do the same extractions with the (M) samples except that to avoid emulsions the shaking must be only moderate and centrifugation should be 10 minutes.
- (m) Into a third 15 ml silylated tube, pipet another 0.5 ml of the sample [(the (I) sample)].
- (n) Blow the (M) and (A) extracts and (I) sample to dryness. Again all solvent must be removed and no moisture picked up. A bath temperature of 40°C is suitable for the (M) and (A) extracts and a temperature of 70°C for the (I) sample.
- (o) Add 0.1 ml of the derivatizing reagent to all (M), (A), and (I) dry samples and standards, vortex, tape the stoppers in place, centrifuge briefly, and place in a 95-100°C oil bath for one hour, all at the same time.
- (p) Cool to room temperature, add methanol to the 1 ml mark.

- (q) Add 1 percent isobutyl alcohol in hexane to the 7 ml mark and mix.
- (r) Add water to the 11 ml mark, shake one minute, and centrifuge 5 minutes.
- (s) Move the upper layer as completely as possible to a fresh 15 ml tube. Move *no* lower layer.
- (t) Repeat the extractions with a second 6 ml of solvent. Combine extracts.
- (u) Blow completely to dryness with nitrogen purge in a 40°C water bath. At the end of this step the temperature can be allowed to rise to 60-70°C.
- (v) Take up residue in exactly 0.5 ml of methanol using vortex mixing and ultrasound to dissolve. In all steps above the (M) samples should be handled first and placed on the LC before handling the (A) or (I) samples. After extraction protect the (M) samples from light as much as possible. If (A) and (I) samples are to be delayed significantly, they should be stored in a freezer at -20°C before adding the methanol.
- (w) Centrifuge and inject 50 ul of the clear phase (M samples) onto the LC with one standard of each type, the unknowns, and finally the second standard, in that order.
- (x) Do the same with the (A) and (I) samples and standards.
- (y) Examine the unknowns for the presence of the (A), (M), and (I) peaks at the same elution time as corresponding standard peaks.
- (z) Average the two standard values for each sample and calculate each unknown as a percent of that standard.
- (aa) A value of 60 percent or more of all three (A, M, and I) is proof that the particular level of ivermectin is present in the original sample.

References

- Egerton. J. R., et. al., *Antimicrob Agents Chemother.*, 1979, 15, 372-378
- Tolan, J. W., et. al., *J. Chromatogr.* 1980, 190, 367-376
- Tway, P. C., Wood, J. S., Downing, G. V., *J. Agric. Food Chem.*, 1981, 29, 1059-1063.

5.036. Detection of Cresylic Acid in Fat

5.036A. Theory

Cresylic acid is used in poultry husbandry as an employee's foot bath to prevent their shoes from becoming a vector for disease. It has a potential to become a contaminant in poultry because of its solubility in fat. Cresylic acid is also slightly soluble in water and is difficult to extract from fat or water with organic solvents. The boiling point and fat solubility of cresylic acid are high enough so that adipose tissue can be safely rendered at 100°C with no significant loss. The rendered fat is dissolved in hexane, extracted with 5 N sodium hydroxide, derivatized with dichloroacetylchloride, extracted with chloroform, and, after separation and evaporation of the chloroform, dissolved in hexane and analyzed by GC-EC.

5.036B. Apparatus

- (a) Gas Chromatograph Hewlett-Packard 5710A (or equivalent) equipped with Electron Capture detector (NI⁶³).
6ft. x 2mm glass column packed with 20% OV-225/20% OV-17 (1 + 1) on 80/100 mesh Chromosorb W-HP.
Argon/Methane carrier gas 95/5 at 50ml/min.
Oven temperature, isothermal at 210°C. Injector temperature 250°C. Detector temperature 350°C.
- (b) Scintillation vial, 20ml Wheaton Scientific No. 98654, or equivalent (with teflon-lined cap).
- (c) Glass stoppered 50ml centrifuge tube, Corning-No. 8424, or equivalent, with #13 standard taper ground glass stopper.
- (d) Serological pipets, disposable, 9", Scientific Products #P5211-2, or equivalent.
- (e) Centrifuge Damon/IEC HN-SII, or equivalent.
- (f) Glass fiber filter, 9.0cm circles, Reeve Angel 934AH, or equivalent.

5.036C. Reagents

- (a) Hexane UV grade, Burdick and Jackson, or equivalent.
- (b) Chloroform, Pesticide grade, Omnisolv CX1059-1, or equivalent.
- (c) Dichloroacetylchloride, Eastman, redistilled, or equivalent.
- (d) 5N NaOH, reagent grade, Fisher Scientific, SO-S-256, or equivalent.
- (e) Toluene, Pesticide grade, Burdick and Jackson, or equivalent.
- (f) Sodium Sulfate anhydrous, Mallinckrodt, analytical grade, or equivalent.

5.036D. Sample Preparation

A sample of adipose tissue is rendered at 100°C, filtered through glass fiber filter, and weighed into a 50ml centrifuge tube.

5.036E. Determination

- (a) Weigh 4.0g rendered fat into a 50ml centrifuge tube and add 10ml UV hexane. Stopper and shake until fat is dissolved.

- (b) Add 1.0ml 5 N NaOH and gently shake for (2) two minutes.
- (c) Add 4.0ml distilled H₂O and gently shake for (1) one minute.
- (d) Centrifuge at 1500 rpm for (2) two minutes and aspirate the organic (hexane) layer.
- (e) Add 10ml hexane UV to the aqueous phase and mix layers by rotating tube end over end (bicycling) for 10 seconds.
- (f) Centrifuge at 1500 rpm for 30 seconds and aspirate the organic (hexane) layer.
- (g) Add 5.0ml chloroform and (1) one drop of Dichloroacetylchloride (DCA) to the aqueous phase and shake vigorously for (1) one minute.
- (h) Add (1) one more drop of DCA and shake vigorously 30 seconds.
- (i) Centrifuge at 1500 rpm for (2) two minutes, and aspirate the aqueous layer.
- (j) Add 2.0g to 3.0g anhydrous sodium sulfate to the organic phase and mix.
- (k) Transfer organic chloroform layer to a 20ml scintillation vial via a disposable pipet, add 1.0ml toluene and evaporate to approximately 200 ul final volume.
- (l) Add 2.0ml hexane UV, mix well and inject 3.0 ul on a Gas Chromatograph (see 5.036B.(a)). An argon/methane carrier gas, 95/5, with a flow rate of 50ml/min will produce a retention time for the cresylic acid dichloroacetate ester of approximately five minutes.

PART 6—NUTRITIONAL ANALYSES

6.001 Determination of Vitamin A

This procedure is applicable for the analysis of total vitamin A (vitamin A plus carotene) in meat and poultry products.

6.001A. Theory

Antimony trichloride forms a color complex with both vitamin A and carotene. Column chromatography is used to separate the vitamin A fraction from that of carotene. The Carr-Price reaction product is used to measure vitamin A at 620 nm. Carotene is estimated by its absorption at 436 nm.

6.001B. Apparatus

All glassware must be amber or low-actinic glassware. Vitamin A is light sensitive; to prevent its destruction this special glassware or subdued lighting must be used.

- (a) Boiling flask: 250 ml
- (b) Condensers: Air-or water-cooled
- (c) Erlenmeyer flasks: 500 ml; 250 ml
- (d) Separatory funnels: 500 ml
- (e) Volumetric pipettes
- (f) Mohr pipette: 10 ml
- (g) Chromatographic tubes: 2.5 cm od \times 25.0 cm with sealed-in disc of medium porosity and with funnel on upper end and stem on lower end, 8 mm od \times 40 mm (available from SGA, Scientific Inc.)
- (h) Ultraviolet lamp: Longwave, 360 nm is recommended. Caution! Vitamin A is destroyed by too intense UV light; use low-intensity lamp.
- (i) Spectrophotometer: Suitable for reading at 620 nm and 426 nm.

6.001C. Reagents

- (a) Potassium hydroxide solution: Dissolve 90 g KOH in 100 ml
- (b) Diethyl ether (Peroxide free)
- (c) Chloroform (H₂O and phosgene free)
- (d) Hexane

(e) Acetone

(f) Nitrogen (oxygen free)

(g) Hyflo Super-Cel

(h) Sea Sorb 43

(i) Isopropanol

(j) Ethanol

(k) Antimony Trichloride Reagent: *Warning! Corrosive Reagent!* Dissolve 114g SbCl_3 in 500 ml of chloroform. It may be necessary to warm the mixture. Filter and store in dry, glass-stoppered standard taper bottle.

(l) Acetone - hexane mixture - 50 ml acetone and 450 ml hexane

(m) Vitamin A Standards:

(1) Weigh approximately 0.5 g of vitamin A Reference Standard into a 50 ml volumetric flask. This is the contents of 3 capsules. Make up to volume with isopropanol. This is the stock solution and must be refrigerated. One gram of Vitamin A Reference Standard contains 100,000 International Units.

(2) Transfer a 2 ml aliquot of the stock solution to a 200 ml volumetric flask and make up to volume with chloroform. (1 ml = approximately 10 IU). This must be refrigerated and prepared fresh on the day of use.

(n) Hyflo Super-Cel: Sea Sorb 43 (1:1) Mix 1 pound of each and store.

(o) B-carotene. Prepare solutions of pure B-carotene in acetone-hexane (1 + 9) just before use for preparation of standard curve (0.6 mg B-carotene is equivalent to one IU Vitamin A).

6.001D. Determination

Protect Vitamin A from strong illumination by working in subdued light or by using low-actinic glassware. From stated label claim, process sufficient sample to contain about 200 IU vitamin A. Frozen products should be first ground by two passes through meat grinder. Grind 200-300 g for sampling. The sample is comminuted in a blender as quickly as possible to prevent oxidation.

(a) Transfer 20 g of sample to a 250 ml boiling flask with a ground glass neck. Use a powder funnel to keep sample from touching neck and wash in with 50 ml of water.

(b) Add 50 ml of ethanol, 10 ml of the KOH solution and a few grains of alundum for boiling. Mix sample, and reflux for one-half hour with occasional shaking. If unsaponified fat is visible at end of digestion period, add another 10 ml aliquot of KOH solution to sample and repeat saponification. Higher KOH: ethanol ratio should be used for all known high fat content samples.

(c) Cool sample, shake with stopper on top to get most of the sample off the sides and then transfer to a 500 ml separatory funnel. Police flask and transfer all washings to separatory funnel.

(d) Add 50 ml of ether and 50 ml of hexane to the separatory funnel. Invert once and release the pressure from the top. Invert once more and release pressure. Sample may now be shaken normally for approximately 1 minute.

(e) Allow the layers to separate and transfer the water layer to the original boiling flask. Use a wire to push the solids through the stopcock.

- (f) Place the ether-hexane layer into a 500 ml Erlenmeyer flask (first extraction).
- (g) Transfer the water layer back into the separatory funnel and extract with 50 ml of ether and 50 ml of hexane.
- (h) Transfer the water layer back into the boiling flask and discard.
- (i) Combine the extracts in the separatory funnel, washing the 500 ml Erlenmeyer flask with ether. Add wash to separatory funnel.
- (j) Add 300 ml of water and gently invert once. Caution! DO NOT SHAKE: VITAMIN A WILL BE LOST.
- (k) Drain water layer into 500 ml Erlenmeyer and wash the ether layer again with 300 ml of water by inverting one time.
- (l) Draw off small portion of water layer into test tube and see if it is neutral to phenolphthalein. If not, wash until neutral, then draw off water layer into 500 ml Erlenmeyer. Discard water washings.
- (m) Draw off the ether layer into a 250 ml Erlenmeyer and place in a hot water bath to drive off the ether.
- (n) Blow a stream of oxygen-free nitrogen onto the sample while boiling. This speeds volatilization and keeps Vitamin A from oxidizing. When dry, cover residue with a few ml of hexane.

6.001E. Column Preparation

Column should be approximately 25.0 cm long and 2.5 cm in diameter. Place a piece of glass wool over the tip of the column, put a one-hole rubber stopper on the column tip and place in a 125 ml suction flask.

- (a) Pour a 1:1 mixture of the absorbents through a powder funnel into the column. Add enough adsorbent to give a height of 12.5 cm and tamp lightly.
- (b) Pull a vacuum and wet column with hexane. Just before the column goes dry, pour the sample into the column and wash lip and flask with hexane and add to column.
- (c) Just before column goes dry, add the eluant (10 percent acetone-hexane mixture) and observe elution of orange band of carotene. (Carotenes pass through column rapidly).
- (d) Collect entire carotene band. When all the carotene is in the 125 ml suction flask, turn off the vacuum and remove the flask.
- (e) Transfer eluate, which has been reduced in vol. by loss of vapor thru H_2O pump to 100 ml vol. flask, dil to vol. with acetone-hexane (1 + 9), stopper and set aside in dark for later analysis.
- (f) Mark this flask "carotene fraction".
- (g) Place second flask under column and continue eluting until all vitamin A comes off column. Observe elution of vitamin A by brief inspections with UV light (Vitamin A fluoresces under UV light). Mark this flask "vitamin A fraction".

6.001F. Photometer Measurement

- (a) Vitamin A fraction

The wavelength is set at 620 nm. Instrument should have at least 15-minute warmup before use.

The instrument is first set to zero on the absorbance scale using a reagent blank. The reagent blank consists of 1 ml chloroform and 3 ml of the $SbCl_3$ solution.

Pipet 1 ml of the standard solution (1 ml = approximately 10 IU) into the cuvette; place cuvette in the photometer, and quickly add 3 ml of the SbCl_3 solution by means of a dispensing pipet. The absorbance needle first goes up quickly, then down quickly and back up to a pause. Take your reading at the point where the needle pauses. The solution is blue, but decolorizes very quickly, so the reading must be taken exactly at the point of the pause. Each standard and each sample should be in duplicate and the average value used for the reading. One ml of sample is added to the cuvette and 3 ml of SbCl_3 solution added quickly. The reading is taken and repeated again using the average value. If reading of sample is too low, take more sample; if too high, make a further dilution.

Sample should read within 5-15 International Units.

(b) Carotene fraction

Calibrate suitable spectrophotometer with acetone-hexane (1 + 9) solns of pure B-carotene as shown by characteristic absorption curve (J. Bio) Chem *144*,21 (1942). Read absorbance at 436 nm. Prep. Calibration chart and convert absorption A of soln to be determined to carotene concentration from chart as mg carotene/ml.

6.001G Calculations:

United States Recommended Daily Allowance (USRDA) = 5000 International Units (IU)

$$(a) \frac{\text{IU vitamin A}}{\text{Serving}} = C(\text{std}) \times \frac{A(\text{sample})}{A(\text{std})} \times \frac{V(\text{sample})}{W(\text{sample})} \times F$$

$C(\text{std})$ = concentration of standard (IU/ml)

$A(\text{sample})$ = absorbance of sample

$A(\text{std})$ = absorbance of standard

V = volume of sample (ml) aliquot

$W(\text{sample})$ = weight (g) of sample in the aliquot

F = serving size converted to the appropriate dimensions (i.e. ounces to grams etc.)

(b) Calculate carotene as units vitamin A per serving

$$\text{IU Vitamin A (as B-carotene) per serving} = C \times \frac{V}{W} \times F \times 1.667$$

C = mg carotene/ml in 100 ml flask

V = volume of sample (100 ml)

W = weight of sample in aliquot (20g)

F = serving size converted to appropriate dimensions (ounces to grams, etc)

1.667 = equivalent of carotene to vitamin A

(c) Total I.U. Vitamin in sample = (a) + (b)

$$\text{USRDA} = \frac{\text{mg Vitamin A}^*}{5000} \times 100 \quad \text{*IU Vitamin A found per serving}$$

Reference

1. Methods of Vitamin Assay, The Association of Vitamin Chemists (1966)
2. Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

6.002 Determination of Vitamin B₁ (Thiamine)

This procedure is applicable to meat or poultry products.

6.002A. Theory

Thiamine occurs naturally in foods either in a free or combined form as a protein complex, or as a pyrophosphoric acid ester, cocarboxylase. A deficiency of thiamine results in beriberi, polyneuritis, neuritis of several peripheral nerves, loss of weight and appetite, etc.

Thiamine or thiamine hydrochloride is rapidly destroyed in alkaline or neutral solutions, however, in acid solutions (pH 3.5), the vitamin can be autoclaved at 120° C for 30 minutes without loss of activity.

The determination of thiamine is based on the oxidation of thiamine to thiochrome, which fluoresces in ultraviolet light. In the absence of other fluorescing compounds, the fluorescence is proportional to the amount of thiochrome present, and therefore the amount of thiamine in a sample.

Thiamine must be freed from interfering substances by elution from an appropriate absorption column.

6.002B. Apparatus

(a) Volumetric flasks: 100 ml

(b) Powder funnels

(c) Chromatographic columns: Base exchange tubes with reservoir at top approximately 35 mm od × 95 mm, adsorption tube mid-section 8 mm od × 145 mm, drawn into a capillary at the bottom 35 mm long which is restricted to permit a flow of 1 ml/minute. (Tubes are commercially available as base exchange tubes).

(d) Volumetric pipets

(e) Erlenmeyer flasks: 125 ml

(f) Standard taper glass-stoppered graduated cylinders

(g) Standard taper glass-stoppered 50 ml centrifuge tubes

(h) Photofluorometer: Fitted with input filter of narrow transmittance range with maximum ca 365 nm and an output filter of narrow transmittance range with maximum at ca 435 nm.

6.002C. Reagents

(a) Anhydrous sodium sulfate, granular

(b) 15 percent NaOH Solution: Dissolve 150 g NaOH in water and dilute to 1 liter.

(c) 1 percent Potassium ferricyanide solution: Dissolve 5 g of K₃Fe(CN)₆ in water and dilute to 500 ml. Stable indefinitely if kept cool and dark in brown bottle.

(d) Alkaline potassium ferricyanide solution - Dilute 3 ml of 1 percent K₃Fe(CN)₆ to 100 ml with 15 percent NaOH solution. Prepare fresh daily and keep out of sunlight.

(e) 0.1 N H₂SO₂ Solution: Dilute 2.8 ml of conc H₂SO₄ to 1 liter with water.

(f) 2.5 M Sodium acetate solution: Dissolve 345 g of NaC₂H₃O₂·3H₂O in water and dilute to 1 liter.

- (g) Isobutyl alcohol: Check fluorescence, should \leq 10 percent of quinine standard.
- (h) Enzyme solution: prepare fresh daily. Dissolve one of the following enzymes in 50 ml of 2.5 M sodium acetate; (1) 2 g Takadiastase, (2) 6 g Prolidase S, or (3) 2 g Mylase P.
- (i) Acid 25 percent Potassium chloride solution: Dissolve 250 g of KCl in water, add 8.5 ml conc HCl, and dilute to 1 liter with water.
- (j) 3 percent KCl Solution: Dilute 25 ml of 25 percent KCl solution to 200 ml with 0.1 N HCl.
- (k) Activated Decalso (Thiochrome Decalso): Add 4 g per column of Decalso to a 400 ml beaker. Wash twice with hot 3 percent KCl (keep KCl in contact with Decalso for 10 minutes), then with hot distilled water till free of chlorides. Check with 1 percent AgNO_3 .
- (l) Stock Thiamine Solution: Dry Thiamine hydrochloride over P_2O_5 for 24 hours. Dissolve 100 mg in 0.01 N HCl and make to 1 liter volume with same. Stable for six months if kept at 5° C in dark bottle.
- (m) Intermediate Thiamine Solution: Dilute 5 ml of stock solution to 100 ml with water.
- (n) Working Thiamine Solution 0.2 $\mu\text{g}/\text{ml}$: Transfer 4 ml of intermediate thiamine solution to a flask containing 75 ml of 0.1 N H_2SO_4 and 5 ml of sodium acetate solution and adjust to 100 ml with water. Make fresh daily.
- (o) Stock Quinine Sulfate Solution: Dissolve 100 mg in 0.1 N H_2SO_4 and dilute to 1 liter with the same solvent. Stable indefinitely in dark brown bottle.
- (p) Working Quinine Sulfate Solution: Dilute 3 ml of stock solution to 1 liter with 0.1 N H_2SO_4 .

6.002D. Procedure:

The sample must be comminuted in a blender before it can be used. To do this, weigh 300 g of sample and 300 g of water into a blender and comminute.

- (a) Weigh 20 g of the slurry (10 g sample) into a 125 ml low-actinic Erlenmeyer.
- (b) Add 7.5 ml of 1 N HCl and 40 ml of distilled water.
- (c) Add 2 drops of caprylic alcohol to prevent foaming and place in autoclave for 15 minutes at 15 lb pressure.
- (d) Cool to room temperature and add 5 ml of the enzyme solution to each sample.
- (e) Incubate for 2 hours at 45° C. At this point, the sample has a pH of 4.5 and is stable for 2 weeks in a refrigerator.
- (f) Bring sample to 100 ml with water and filter through a Whatman #4 paper.
- (g) Prepare columns using the activated Decalso. Wash twice with hot 3 percent acidified KCl and then with hot distilled water until clear of chlorides. Wash once with distilled water to cool columns. Always keep water above the Decalso in the column.
- (h) Pass 50 ml of the filtered sample through the column. This may have to be done with two 25 ml aliquots. After sample has passed through the column, wash twice with distilled water to remove any extraneous matter in column.
- (i) Elute column with 25 ml (10, 10, 5 ml aliquots) of 25 percent acidified KCl solution into a ground glass graduated cylinder. You can stop at this point and refrigerate.
- (j) A standard of 0.2 $\mu\text{g}/\text{ml}$ should be prepared and 25 ml passed through the column the same as the sample. If solution in graduate is not 25 ml, make the 25 ml with water.

Only two reaction vessels can be used at a time. Into one 50 ml centrifuge tube, pipette 5 ml of standard and into the other pipette 5 ml of sample. To each tube add 3 ml of alkaline ferricyanide solution, mix gently, then add 20 ml of isobutyl alcohol. Place on the shaker and shake for exactly 90 seconds. Centrifuge at 2500 rpm for 60 seconds. Remove and discard lower aqueous phase using syringe (approximately 9 ml). Add a heaping spatula (ca 2g) of sodium sulfate to the isobutyl layer and centrifuge for 60 seconds.

Adjust meter reading on photofluorometer to 60 using quinine sulfate as the set point solution.

Pour standard (0.2 µg/ml) into cuvette and read. Add 3 drops of (1:1) HCl, mix and read standard blank. Do the same with the samples.

6.002E. Calculations:

United States Recommended Daily Allowance (USRDA) = 1.5 mg

$$\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{std}} - A'_{\text{blank}}} \times C_{\text{std}} \times \frac{\text{Df}}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{1.5} \times 100$$

A_{sample} = Fluorescence of sample

A_{blank} = Fluorescence of sample blank

A'_{blank} = Fluorescence of standard blank

A_{std} = Fluorescence of standard

C_{std} = Concentration of standard (0.2 µg/ml)

Df = Dilution factor, if above dilutions are used, the factor

$$\frac{25 \text{ ml}}{50 \text{ ml}} \times \frac{100 \text{ ml}}{10 \text{ g}} = 5 \text{ ml/g}$$

F = Serving size converted to the appropriate dimensions (i.e. ounces to grams, etc.)

1000 = micrograms per milligram

Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966)

6.003 Determination of Vitamin B₂ (Riboflavin)

This method is applicable to meat and poultry products. The sample preparation is similar to that for vitamin B₁ and a suitable aliquot from the B₁ sample prep may be used in this determination.

6.003A. Theory

Riboflavin is a naturally occurring pigment that has wide distribution in plant and animal cells. In living cells riboflavin is usually combined with phosphoric acid or with phosphoric acid and adenylic acid (adenosine monophosphate), both of which may be combined with specific proteins.

Because it may be combined with proteins, it is necessary to treat natural products with acid or enzymes to free the riboflavin from its protein combination and make it available for extraction.

Riboflavin fluoresces in light of wave length 440 to 300 nm. The intensity of fluorescence is proportional to the concentration of riboflavin. The riboflavin is determined by the difference between its fluorescence before and after chemical destruction. All readings are taken on one aliquot to compensate for the variations due to the presence of interfering substances. Interfering substances that fluoresce may be removed by adsorption chromatography or oxidation or both.

6.003B. Apparatus:

All glassware should be low-actinic.

(a) Test tubes: 18 mm od × 150 mm

(b) Funnels: 2-3 inches diameter

(c) Erlenmeyer flasks, low-actinic: 125 ml

(d) Filter Paper: Whatman No. 541

(e) Volumetric flasks: 25 ml, 100 ml

(f) Volumetric pipettes: Assorted sizes

(g) Photofluorometer: Use fluorometer suitable for accurately measuring fluorescence of solutions containing riboflavin in concentrations of 0.05 - 0.2 µg/ml. Instrument should be fitted with input filter of narrow transmittance range with maximum at ca 440 nm and output filter of narrow transmittance range with maximum at ca 565 nm.

6.003C. Reagents:

(a) Sodium acetate 2.5 M: Dissolve 340 g of sodium acetate in water and make up to 1 liter.

(b) Enzyme solution: Prepare fresh daily. Dissolve one of the following enzymes in 50 ml of 2.5 M sodium acetate; (1) 2 g Takadiastase, (2) 6 g Prolidase S, or (3) 2 g Mylase P.

(c) Glacial acetic acid.

(d) 1 N Hydrochloric acid: Add 83 ml of concentrated HCl to water and make up to 1 liter.

(e) 4 percent KMnO₄ Solution: Dissolve 4 g of KMnO₄ in water and make up to 100 ml. Stable for one week.

(f) Caprylic alcohol

(g) 3 percent Hydrogen peroxide

(h) Sodium Fluorescein Solution ($0.05\mu\text{g}/\text{ml}$). Weigh 0.05 g of sodium fluorescein into a 1 liter volumetric and make to volume with water. Take a 1 ml aliquot and make to 1 liter with water, ($0.05\mu\text{g}/\text{ml}$).

(i) Sodium hydrosulfite (Dithionite): High purity and stored to avoid undue exposure to light and air.

Riboflavin standards ($25\mu\text{g}/\text{ml}$): (1) Weigh accurately 25 mg U.S.P. Reference standard riboflavin dried over P_2O_5 into a 1 liter volumetric flask. Add 700 ml of water, 1.2 ml of glacial acetic acid and warm to aid solution. Cool and make up to volume with water. Preserve under toluene, protect from light in a refrigerator. Stock solution good for six months.

(j) Riboflavin Standard A ($5\mu\text{g}/\text{ml}$): Dilute 20 ml of stock solution to 100 ml with water. Preserve under toluene, protect from light in a refrigerator. Good for two weeks.

Riboflavin Working Standard B ($0.5\mu\text{g}/\text{ml}$): Dilute 10 ml of stock solution A to 100 ml using 7.5 ml of 1 N HCl, 5 ml of 2.5 M NaOAc, and make to volume with water. Store under toluene, protect from light in a refrigerator. Stable for one week.

6.003D. Procedure:

The sample must be comminuted in a blender before it can be used. To do this, weigh 300 g of sample and 300 g of H_2O into a blender and comminute.

(a) Weigh 20 g of the slurry into a 125 ml low-actinic Erlenmeyer.

(b) Add 7.5 ml of 1 N HCl and 40 ml of distilled water.

(c) Add 2 drops of caprylic alcohol to prevent foaming and autoclave for 15 min at 15 lb pressure. The 20 g sample weighed consists of 10 g sample and 10 g water. Keep this in mind for calculations.

(d) Cool to room temperature and add 5 ml of the enzyme solution to each sample. Incubate for 2 hrs at 45°C . At this point, the sample has a pH of 4.5 and is stable for two weeks in a refrigerator.

(e) Transfer sample to a 100 ml volumetric flask and bring to volume with water. *From this sample preparation* aliquots for both riboflavin and thiamine determinations may be taken.

(f) Filter sample through Whatman #4 or #541 paper. Transfer a 15 ml aliquot to a 25 ml low-actinic volumetric flask.

(g) Add 1 ml of glacial acetic acid, 1 ml of a 4 percent KMnO_4 solution and allow to stand exactly 2 minutes.

(h) Add 5 ml of 3 percent H_2O_2 and mix thoroughly. Add two drops of caprylic alcohol and bring to volume.

(i) Using the $0.05\mu\text{g}/\text{ml}$ sodium fluorescein, adjust the instrument to give a deflection of 50 on the meter. Check adjustment before reading each series.

(j) Darken lights in the room. Transfer a 10 ml aliquot to a test tube.

(k) Pour sufficient sample from the test tube to the cuvette and read fluorescence.

(l) Pour sample back into the test tube. Add a 1 ml aliquot of the Riboflavin working standard, mix and read in fluorometer.

(m) Pour back in test tube, add ca 10 mg of sodium hydrosulfite, mix and read fluorescence.

6.003E. Calculations:

United States Recommended Daily Allowance (USRDA) = 1.7 mg

$$\frac{A - 1.10C}{1.10B - A} \times \frac{\text{Ribo. increment}}{10} \times \frac{\text{Df}}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg per serving}}{1.7} \times 100$$

A = sample absorbance

B = sample + standard absorbance

C = absorbance after addition of sodium hydrosulfite

10 = sample aliquot (ml).

1000 = micrograms/milligram

Df = dilution factor—if above dilution are used factor 16.7 ml/g

1.10 = Decimal equivalent of 11/10 (10 ml sample aliquot plus 1 ml riboflavin working standard)

F = Serving size converted to the appropriate dimensions (i.e. ounces to grams etc.)

Ribo. increment = 0.5 μ g/ml—if method is followed

Reference

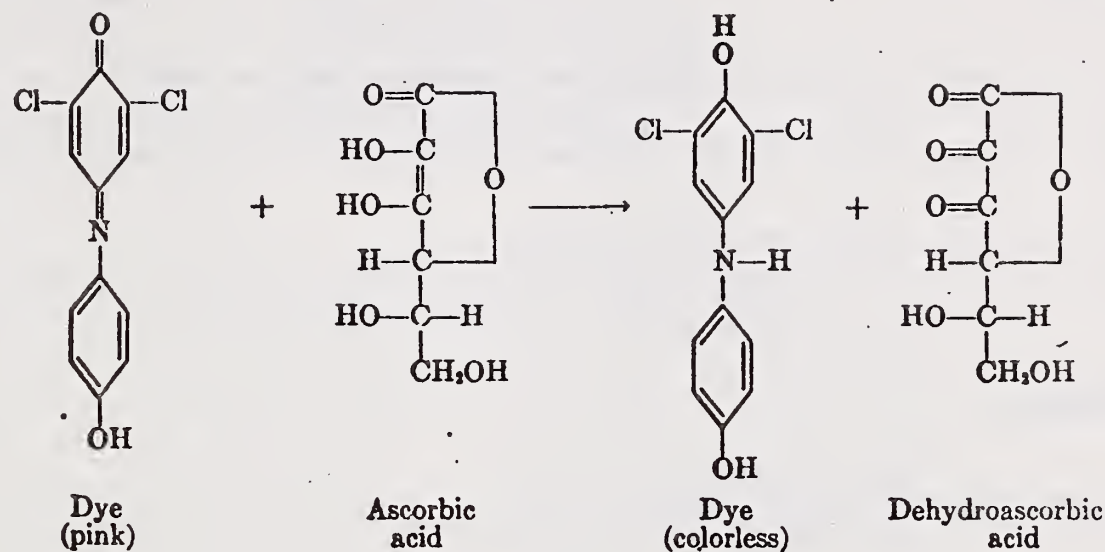
Methods of Vitamin Assay, The Association of Vitamin Chemists (1966)

6.004 Determination of Vitamin C (Ascorbic Acid) Titration Procedure

This method is applicable to the determination of reduced ascorbic acid and its sodium salt, and d-isoascorbic acid (erythorbic acid) and its sodium salt in meat and poultry products.

6.004A. Theory

The oxidation reduction properties of ascorbic acid are widely used as the primary reaction for the determination of vitamin C. The reducing property of vitamin C is measured by using a suitable oxidizing agent such as 2,6-dichlorophenol indophenol. The following equation illustrates this reaction.



In this procedure the dye is made up in a dilute sodium bicarbonate solution and is blue, but in acid medium the dye is pink.

6.004B. Apparatus

- (a) Blender
- (b) Funnel, 3 inch diameter
- (c) Filter paper
- (d) Volumetric pipette 25 ml
- (e) Burette 50 ml capacity
- (f) Stop watch
- (g) Magnetic stirrer and stirring bars

6.004C. Reagents:

(a) Indophenol Dye: Add 105 mg NaHCO_3 and 125 mg 2,6-dichlorophenol indophenol sodium salt to 125 ml distilled water. Shake until dissolved and dilute to 500 ml. Store in amber bottle in refrigerator. Prepare weekly.

(b) Ascorbic Acid Stock Solution: Add 100 mg ascorbic acid to 1 ml glacial acetic acid. Dilute to 100 ml with distilled water (make fresh daily).

(c) Ascorbic Acid working solution (make just before use): Add 4 ml of stock soln to 96 ml distilled water.

(d) Metaphosphoric Acid: Dilute 15 g metaphosphoric acid in 40 ml glacial acetic acid to 500 ml with distilled water.

The 2,6-dichlorophenol indophenol dye is reduced equally well by the enediol groups of ascorbic acid, sodium ascorbate, isoascorbic acid and sodium isoascorbate, so that the method below is applicable to all four compounds and cannot distinguish among them.

6.004D. Procedure:

(a) Blend 10 g of product with 95 ml metaphosphoric acid, Reagent (d), and filter.

(b) Titrate 25 ml aliquot of filtrate with the indophenol dye soln until pink color remains at least 30 seconds.

(c) Titrate 25 ml aliquot ascorbic acid working solution.

(d) Titrate 25 ml reagent (d) as a reagent blank.

6.004E. Calculations:

$$\text{ppm ascorbic acid} = \left[\frac{(A - B)C}{D} \right] 1000$$

United States Recommended Daily Allowance (USRDA) = 60 mg

$$\text{mg Vitamin C per serving} = \left[\frac{(A - B)C}{D} \right] F$$

$$\% \text{ USRDA} = \frac{\text{mg vitamin C/serving}}{60} \times 100$$

A = ml sample aliquot titration

B = ml reagent blank titration

C = mg ascorbic acid per ml dye = $\frac{1}{\text{ml standard aliquot titration}}$

D = 2.5g sample wt.

F = Serving size converted to the appropriate dimensions (i.e. ounces to grams, etc.)

NOTES:

1. Ascorbic acid solutions are unstable and the presence of oxidizing agents in the meat (e.g., nitrites) cause the solution to decompose rapidly, therefore, conduct the analysis for ascorbic acid and its related compounds as rapidly as possible.

2. A blank value can be obtained by analyzing untreated meat in the same way to determine the ascorbic acid equivalent of the natural reducing substances present in meat. (For ground beef this value is 25 ppm calculated as ascorbic acid.)

3. The following compounds may be analyzed by this procedure:

Ascorbic acid also known as "Vitamin C"

Sodium ascorbate

d-isoascorbic acid also known as Erythorbic acid

Sodium d-iso ascorbate also known as sodium erythorbate

4. If possible standardize with the ascorbate compound to be analyzed in the meat sample. The following are equivalents between the various compounds:

1 part Ascorbic Acid	=	1.000 parts Erythorbic Acid
1 part Ascorbic Acid	=	1.124 parts Sodium Ascorbate
1 part Ascorbic Acid	=	1.124 parts Sodium Erythorbate
1 part Sodium Ascorbate	=	1.000 parts Sodium Erythorbate
1 part Erythorbic Acid	=	1.124 parts Sodium Erythorbate

Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966)

6.005 Determination of Vitamin C (Ascorbic Acid) Spectrophotometric

6.005A. Apparatus

- (a) Blender
- (b) Volumetric flasks: 25 ml, 50 ml
- (c) Volumetric pipettes: 3 ml, 5 ml, 10 ml
- (d) Spectrophotometer: Suitable for reading at 515 nm
- (e) Erlenmeyer flasks: 250 ml
- (f) Funnel: 3 inches diameter
- (g) Filter paper

6.005B. Reagents

- (a) Sodium hydroxide 2 N: Dissolve 20 g NaOH in 600 ml of water and make to 1 liter.
- (b) Citrate buffer solution: Dissolve 117.6 g of citric acid in 560 ml of 2 N NaOH and make up to 1 liter with water. Keep refrigerated.
- (c) Phosphoric Acid-Meta, 6 percent: Fisher A-240 or equivalent. Stable for one week at 4° C.
- (d) Ascorbic acid: Merck 00845 - 61288 or equivalent.
- (e) 2,6-Dichlorindophenol Sodium salt, Fisher S-286 or equivalent: Dissolve 100 mg of dye in boiled distilled water and make up to 100 ml. Take 4 ml aliquot of this solution and make up to 250 ml with water. Keep refrigerated. Prepare weekly.
- (f) Vitamin C Standard A: Weigh 100 mg of Ascorbic acid into a 100 ml plastic beaker. Add 50 ml of 6 percent m-HPO₃ and dissolve. Transfer to a 100 ml volumetric flask and make up to volume with water.
- (g) Vitamin C Standard B: Transfer a 5 ml aliquot of solution A into a 100 ml volumetric flask, and make up to volume with 3 percent m-HPO₃.

6.005C. Procedure

- (a) Weigh 300 g of sample into beaker and transfer to blender
- (b) Weigh 300 g of 6 percent m-HPO₃ into beaker and transfer to blender. Commminute the sample with the m-HPO₃ present. This keeps the ascorbic acid from oxidizing.
- (c) From this mixture, weigh 20 g and transfer to a 100 ml volumetric flask.
- (d) Add 40 ml of 6 percent m-HPO₃, and make up to volume with H₂O. This gives a 3 percent solution of m-HPO₃.
- (e) Filter the solution into a 250 ml Erlenmeyer.
- (f) From the clear solution, take an appropriate aliquot (10 ml) and transfer to a 25 ml volumetric flask.
- (g) Add 5 ml of buffer solution and make up to volume with 3 percent m-HPO₃.

- (h) The final concentration of the solution should be 3 $\mu\text{g/ml}$ and be in a volume of 25 to 50 ml, but no more.
- (i) Prepare standard solution C to be read on the spectrophotometer by transferring a 3 ml aliquot of solution B to a 50 ml volumetric flask.
- (j) Add 10 ml of buffer solution and make up to volume with 3 percent m-HPO_3 .
- (k) Prepare the blank by transferring 5 ml of buffer solution to a 25 ml volumetric flask and make up to volume with 3 percent m-HPO_3 .

The samples, the standard, and the blank should be run in duplicate, so enough test tubes to accommodate these should be placed in the test tube rack.

The readings on the spectrophotometer should be taken at 515 nm. Place distilled water in one of the test tubes and set the instrument to 100 percent transmittance.

- (l) Into each of the test tubes, pipet 3 ml of dye.
- (m) Pipet 3 ml of standard solution C to the dye in one of the test tubes and read. The addition should be made rapidly. Color changes from blue to pink.
- (n) Add a few crystals of ascorbic acid to the test tube and read. The solution is reduced and becomes colorless. Do this to a duplicate tube and record readings.
- (o) Pipet 3 ml of sample to tube and read. Reduce with a few crystals of ascorbic acid and read. Do this to duplicate tube and record readings.
- (p) Pipet 3 ml of blank solution to each of two test tubes.
- (q) To one of the test tubes, add a few crystals of ascorbic acid; to the other tube, add nothing.
- (r) Set the blank with the ascorbic acid to the reading obtained when the standard was reduced (e.g. 0.013) and then read the other blank (e.g. 0.357).
- (s) This is the corrected reading and is substituted for the reduced reading. Do this for all the reduced readings and record the corrected reading.

Example

<u>Std. Solution C</u>	<u>Duplicate</u>			
0.201	0.201	=	0.201	unreduced standard reading
0.013	0.009	=	0.011	reduced standard reading
0.188	0.192	=	0.190	corrected standard reading
<u>Sample Solution</u>			(duplicate readings)	
0.284	0.292	=	0.288	unreduced sample reading
0.018	0.018	=	0.018	reduced sample reading
0.266	0.274	=	0.270	corrected sample reading

6.005D. Calculations:

United States Recommended Daily Allowance (USRDA) = 60 mg

$$R \times K \times \frac{Df}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{60} \times 100$$

R = (Corrected dye blank — corrected sample reading)
(Corrected dye blank obtained in step (r)).

$$K = \frac{\text{concentration of standard}^*}{(\text{corrected dye blank} - \text{corrected standard reading})}$$

* Concentration of standard is 9.0 μg if method is followed.

$$D_f = \frac{10 \text{ g}}{100 \text{ ml}} \times \frac{10 \text{ ml}}{25 \text{ ml}} \times 3 \text{ ml} = 0.12 \text{ g} = 120 \text{ mg}$$

F = Serving size converted to the appropriate dimensions (i.e. ounces to grams, etc.)

Reference

Methods of Vitamin Assay. The Association of Vitamin Chemist (1966)

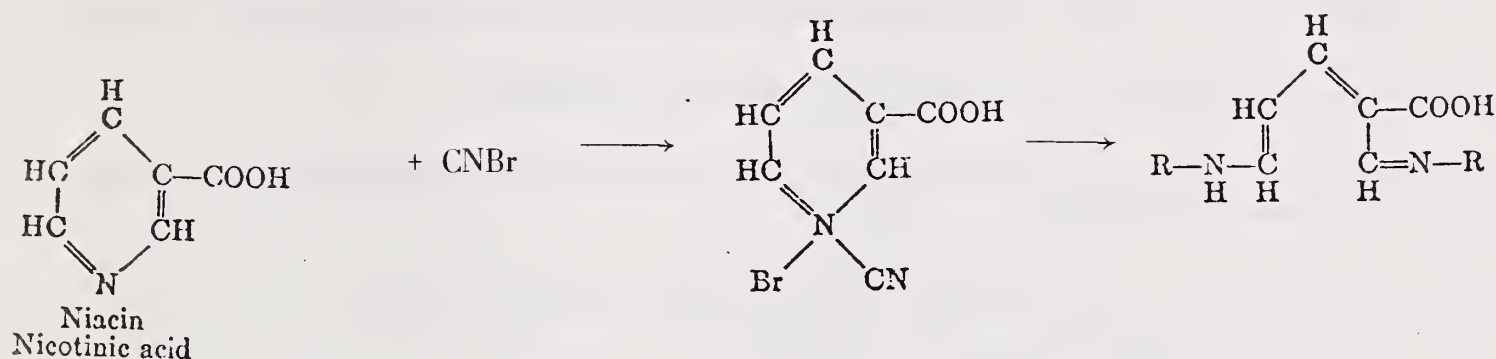
6.006 Determination of Niacin (Nicotinic Acid) and Niacinamide (Nicotinamide)

This method is applicable to the determination of total Niacin or Niacinamide in meat and poultry products.

Added Niacin may be determined with method 6.007.

6.006A. Theory

Niacin reacts chemically the same as pyridine modified by a carboxyl group in the 3 position. Niacin reacts with cyanogen bromide to give a pyridinium compound which rearranges to form derivatives that will couple with aromatic amines, giving colored compounds.



6.006B. Apparatus

- (a) Autoclave
- (b) Spectrophotometer suitable for reading at 450 nm
- (c) Volumetric pipettes: 5 ml, 40 ml
- (d) Volumetric flask: 50 ml, 250 ml
- (e) Glass syringe
- (f) Erlenmeyer flasks: 1 liter

6.006C. Reagents

- (a) Sulfuric acid, 1 N: Dilute 27.8 ml conc. H₂SO₄ to 1 liter.
- (b) Sodium hydroxide, 50 percent ACS reagent, or dissolve 50 g NaOH in 50 ml H₂O
- (c) Bromocresol green indicator: Dissolve 0.1 g tetrabromo-m-cresolsulfonphthalein in 3 ml 0.05N NaOH and dilute to 100 ml. (Yellow 3.8–5.4 blue.)
- (d) Ammonium sulfate, (NH₄)₂SO₄: Reagent grade, crystal
- (e) Nicotinic acid stock solution: Dissolve 0.0500 g U.S.P. Nicotinic Acid Reference Standard in absolute ethanol, dilute to 500 ml and refrigerate in low-actinic glass. Dilute 2 ml to 50 ml with water for standard solution: 1 ml = 4 µg nicotinic acid

(f) Ammonium hydroxide: Dilute 5 ml conc NH_4OH to 250 ml

(g) Sulfanilic acid: Mix 10 g $\text{NH}_2\text{C}_6\text{H}_4\cdot\text{SO}_3\text{H}$ with 85 ml dist water and dissolve by adding 1 ml increments of conc NH_4OH . Adjust pH of solution to 4.5 with HCl and filter.

(h) Hydrochloric acid: Mix 1 part HCl and 5 parts H_2O

(i) Cyanogen bromide solution: Caution—Hood and rubber gloves! Weigh 50 g CNBr by difference into 2 liter Erlenmeyer flask and dissolve in 450 ml warm (40°C) distilled water. Transfer to 500 ml volumetric flask, make to the mark, and store in a glass-stoppered bottle.

6.006D. Procedure

(a) Weigh 28.35 g (1 ounce) of meat sample into a 1 liter Erlenmeyer flask and add 200 ml 1 N H_2SO_4 .

(b) Autoclave 30 minutes at 15 psi (121°C) and cool to room temperature.

(c) Add 10 ml to 50 percent NaOH and adjust to pH 4.5 with further dropwise additions, using pH meter or bromocresol green as external indicator.

(d) If product contains bran, hold at pH 13 for 15 min before adjusting to pH 4.5.

(e) Adjust volume to 250 ml and filter.

(f) Pipet 40 ml filtrate into 50 ml volumetric flask containing 17 g ammonium sulfate, mix, dilute to mark, and filter.

(g) Pipet 40 ml standard (1 ml = $4\text{ }\mu\text{g}$) into 50 ml volumetric flask containing 17 g ammonium sulfate, mix, dilute to mark.

(h) Use a pair of cuvettes for each sample and standard: pipet 1 ml filtrate into each and add 5 ml distilled water to one of each pair (the blank).

(i) Warm up spectrophotometer and set wavelength at 450 nm.

(j) Develop each chromophore and read absorbance before proceeding with the next cuvette: use blank to zero instrument, then read the sample.

(k) To the blank, add 0.5 ml ammonium hydroxide (calibrated dropper) and mix, add 2 ml sulfanilic acid (pipette) and mix, add 0.5 ml hydrochloric acid (calibrated dropper) and mix. Set instrument to zero absorbance within 2-3 minutes.

(l) To the sample (or standard) add 0.5 ml ammonium hydroxide and mix, add 5 ml cyanogen bromide solution (all glass syringe) mix, and let stand 30 seconds. Add 2 ml sulfanilic acid and mix, add 0.5 ml hydrochloric acid and mix, and read absorbance within 2-3 minutes.

6.006E. Calculations:

United States Recommended Daily Allowance (USRDA) = 20 mg

$$\frac{A_{\text{sample}}}{A_{\text{std}}} \times \frac{35.27}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{20} \times 1000$$

A_{sample} = absorbance of sample

$A_{\text{std.}}$ = absorbance of standard

$$35.27 = 3.2 \mu\text{g/ml} \times \frac{50 \text{ ml}}{40 \text{ ml}} \times \frac{250 \text{ ml}}{28.35 \text{ g}}$$

F = serving size converted to the appropriate dimensions (i.e. ounces to grams, etc.)

1000 = micrograms per milligram

Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966)

6.007 Rapid Determination of Added Niacin or Niacinamide

This procedure deals with added niacin, not total niacin. Method 6.006 should be used to determine total niacin (bound plus added).

6.007A. Apparatus

- (a) Volumetric flasks: 100 ml, 50 ml
- (b) Volumetric pipettes: 1 ml, 5 ml, 10 ml
- (c) Funnel
- (d) Filter Paper
- (e) Spectrophotometer suitable to read at 450 nm.

6.007B. Reagents

- (a) Niacin Stock Solution: dissolve 0.100 g U.S.P. Niacin Reference Standard in absolute ethanol, dilute to 1 liter and refrigerate in low actinic glass. Dilute 2 ml to 50 ml with water for working standard solution (1 ml = 4 μ g).
- (b) Cyanogen Bromide Solution: (Caution: use hood and rubber gloves). Weigh 50 g CNBr by difference into 2 liter Erlenmeyer and dissolve in 450 ml warm (40° C) dist water. Transfer to 500 ml volumetric flask, make to the mark, and store in a glass stoppered bottle.
- (c) Aniline Solution: Dilute 2.5 ml aniline to 100 ml with distilled water.

6.007C. Determination

- (a) Dilute 5 g sample into 1 liter volumetric with distilled water, mix well and filter.
- (b) Pipet 10 ml aliquots (or other suitable aliquots) into two separate 50 ml volumetric flasks.
- (c) Run niacin standard solution in parallel (2 ml = 8 μ g).
- (d) Add 5 ml CNBr solution and 1 ml aniline solution to one sample and one standard flask, dilute to volume with distilled water.
- (e) Dilute the second sample flask and the second standard flask to volume with distilled water. These are the sample blank and the standard blank.
- (f) Measure the absorbance at 450 nm, 20 minutes after the addition of reagents. Use the sample blank to zero the spectrophotometer.
- (g) Read the absorbance of the sample solution.
- (h) Re-zero the spectrometer with the standard blank.
- (i) Read the absorbance of the standard solution.

6.007D. Calculations

Compare standard and sample by proportion

$$\% \text{ Niacin} = \frac{\text{Abs}_{\text{sample}} (A)}{\text{Abs}_{\text{standard}} (B)} (10^{-4})$$

A = Micrograms of niacin in standard aliquot (i.e. 2 ml = 8.0 µg).

B = Weight of sample in final aliquot in µg.

10^{-4} = Converts micrograms to percent.

Abs = Absorbance

NOTE: (1) Rapid Qualitative Test

Pour any convenient equal amounts of the initial sample dilution into separate beakers. To one add about 5 ml CNBr soln and 1 ml aniline solution, mix well. A positive test is an intense yellow color produced in this aliquot as opposed to the portion with no reagents added.

(2) High protein samples, such as soya and mustard, tend to get hazy on standing. This haze is not filterable. Therefore, apparent high recoveries are obtained. In evaluating the results, disregard percentages below 0.1 percent even though this method can detect levels less than 0.1 percent.

(3) Due to the colloidal nature of non-fat dry milk, this method will not work quantitatively on that type of sample.

(4) The following values represent the average natural niacin content of fresh meat; analytical findings that more than double these values are to be considered in violation.

Beef	45 ppm
Pork	50 ppm
Veal	70 ppm

The levels of niacin in processed meats may be found in the "Composition of Foods," Agriculture Handbook, No. 8.

6.008 Determination of Iron and Calcium by Atomic Absorption

This procedure is applicable to the determination of iron and calcium in meat and poultry products.

6.008A. Apparatus:

- (a) Atomic absorption spectrophotometer—establish parameters recommended by the instrument manufacturer.
- (b) Erlenmeyer flask—125 ml
- (c) Volumetric pipettes—5, 10, 20, 25, 30, 40, and 50 ml
- (d) Volumetric flasks—100, 250, 1000 ml
- (e) Hood-suitable for use with perchloric acid
- (f) Blender

6.008B. Reagents:

- (a) Nitric Acid
- (b) Perchloric Acid, 70 percent
- (c) Calcium standards

(1) Stock solution—Dissolve 1.249 g of CaCO_3 in a minimum amount of 3 N HCl and dilute to 1 liter. Dilute 50 ml to 1 liter (25 $\mu\text{g}/\text{ml}$).

(2) Working standards—0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 $\mu\text{g Ca}/\text{ml}$ containing 1 percent lanthanum - to 250 ml volumetric flasks add 0, 5, 10, 20, 30, 40, and 50 ml of calcium stock solution (25 $\mu\text{g}/\text{ml}$). Add 50 ml of the 5 percent lanthanum stock solution and dilute to 250 ml. (Commercially prepared calcium standards are available.)

- (d) Iron Standards

(1) Stock solution—Dissolve 1.000 g pure iron wire in approximately 30 ml 6 N HCl with boiling. Cool, dilute 25 ml to 1 liter (25 $\mu\text{g}/\text{ml}$).

(2) Working standards—0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 $\mu\text{g Fe}/\text{ml}$ - Use the dilutions recommended for calcium. *Omit the lanthanum solution.* (Commercially prepared standards are available.)

(e) Lanthanum 5 percent stock solution. Wet 58.65 g of La_2O_3 with distilled water. Add 250 ml concentrated HCl very slowly until the material is dissolved. Dilute to 1 liter with distilled water.

6.008C. Procedure:

- (a) Comminute sample in a blender. Transfer 2 g of sample to a 125 ml Erlenmeyer flask.
- (b) Place three boiling beads into the flask. Add 15 ml of nitric acid to the sample and boil for a few minutes. This oxidizes the sample and reduces the possibility of explosions.
- (c) Remove sample from hot plate, cool, and add 5 ml of perchloric acid.
- (d) Place sample on the hot plate and boil until fumes of perchloric acid appear.

(e) If sample chars at this point, remove from heat, cool and add a few drops of nitric acid.

(f) Place sample on the hot plate and continue heating until fumes of perchloric acid appear. Continue heating for one minute and then remove from hot plate. (CAUTION: NEVER ALLOW THE SAMPLE TO GO TO COMPLETE DRYNESS.)

(g) When sample has cooled, transfer the clear liquid to a 100 ml volumetric flask, add 5 ml of nitric acid and make to volume. This is Solution A, which is used for the iron determination.

(h) For the calcium determination, transfer a 10 ml aliquot of Solution A to a 100 ml volumetric flask, add 20 ml of a 5 percent Lanthanum solution and make to volume. This solution is used for the calcium determination using a 1 percent Lanthanum solution in 5 percent (v/v) HCl to zero the instrument.

6.008D. Determination:

Read ≥ 4 standard solutions within the analytical range before and after each group of 6-12 samples. Flush burner with water between samples and reestablish 0 absorption point. Prepare a calibration curve from the average of each standard before and after sample group. Read concentration of sample from plot or if applicable read directly from instrument.

6.008E. Calculations:

United States Recommended Daily Allowance (USRDA): (calcium) 1.0g

(Iron) 18 mg

$$\text{mg/serving} = \frac{\text{ppm}}{W_t \times 1000} \times F$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{\text{RDA}} \times 100$$

ppm = concentration ($\mu\text{g/ml}$) of element

W_t = weight of sample in final dilution (g/ml)

1000 = microgram per milligram

F = serving size converted to the appropriate dimensions (i.e. ounces to grams, etc.)

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition

6.009 Determination of Iron, Spectrophotometric

This procedure is applicable to the determination of iron in meat and poultry products.

6.009A. Apparatus:

- (a) Vycor crucible: 50 ml
- (b) Volumetric flasks: 25, 100, 500, 1000 ml
- (c) Volumetric pipettes: 2, 5, 10, 20, 30, 40 ml
- (d) Funnel
- (e) Filter paper
- (f) Spectrophotometer suitable for reading at 510 nm.

6.009B. Reagents:

- (a) Hydrochloric Acid: Concentrated, reagent grade
- (b) Standard Iron Solution: Dissolve 3.512 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ in water, add 2 drops HCl, and dilute to 500 ml.
- (c) Working Iron Standard: Dilute 10 ml standard iron solution to 1 liter (1 ml = 0.01 mg Fe).
- (d) Hydroxylamine hydrochloride: Dissolve 10.0 g $\text{NH}_2\text{OH} \cdot \text{HCl}$ in H_2O and dilute to 100 ml.
- (e) Buffer Solution: Dissolve 8.3 g anhydrous NaOAc (dried at 100° C) in H_2O , add 12 ml glacial acetic acid and dilute to 100 ml.
- (f) Dipyrldyl Solution: Dissolve 0.100 g alpha, alpha-dipyrldyl in H_2O and dilute to 100 ml. (Stable several weeks in refrigerator.)

6.009C. Standard Curve:

Add 0, 10, 20, 30 and 40 ml of the working standard plus 2 ml HCl in 100 ml volumetric flask, dilute to volume. Take 10 ml aliquots and proceed as outlined in determination, beginning with step (f) "add 1 ml $\text{NH}_2\text{OH} \cdot \text{HCl}$. . ." Plot absorbance vs mg Fe/ml.

6.009D. Determination:

- (a) Weigh 10.0 g ground sample into a 50 ml vycor crucible and spread in a thin layer on inside walls of the crucible.
- (b) Dry in a 125° C oven and then ash in muffle furnace at 550-600°C. Continue ashing until practically C-free.
- (c) Cool, moisten ash with distilled water, add 5 ml HCl and evaporate to dryness on steam bath.
- (d) Take up residue in 2.0 ml HCl, heating 5 minutes on steam bath, and rinse through filter into 100 ml volumetric flask. Dilute to volume with distilled water.
- (e) Pipette 10 ml aliquot into a 25 ml volumetric flask, set up a reagent blank with 10 ml distilled water plus 2 ml HCl and one or two standards using 10 ml aliquots of iron standards used in preparing working standard curve.

(f) Add 1 ml $\text{NH}_2\text{OH}\cdot\text{HCl}$ solution, mix thoroughly by swirling, add 5 ml buffer solution followed by 2 ml dipyrldyl solution and dilute to volume.

(g) Transfer solutions to cuvettes and read optical density in spectrophotometer at 510 nm.

6.009E. Calculations:

United States Recommended Daily Allowance (USRDA) = 18 mg

$$\text{mg/serving} = \frac{A \cdot B \cdot F}{W_t}$$

A = concentration of iron (mg/ml) from standard curve

B = final volume (ml)

F = serving size converted to the appropriate dimensions (i.e. ounces to grams, etc.)

W_t = weight (g) of sample aliquot in final dilution.

Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

6.010 Determination of Calcium, Titrimetric Procedure

This procedure is applicable to the determination of calcium or bone in meat and poultry products.

6.010A Theory

Calcium is solubilized by acid hydrolysis forming calcium ion. The resultant hydrolyzate is diluted to a specific volume and an aliquot reacted with excess EDTA in alkaline media in the presence of cyanide and a hydroxy naphthol blue indicator. EDTA readily forms a chelated complex with the calcium ion. Excess EDTA is then titrated with calcium carbonate to a permanent purple end point. If phosphates are present they must be removed by passing an aliquot through an ion exchange column before the final titration steps.

6.010B Apparatus

- (a) Laboratory fume hood
- (b) pH meter (Orion Model 701), or equivalent
- (c) 10 ml buret
- (d) 300 ml tall form beaker
- (e) 400 ml beaker
- (f) 200 and 250 ml volumetric flasks
- (g) 20 ml pipet
- (h) Magnetic stirrer (Corning PC-353), or equivalent
- (i) Hot plate
- (j) Filtration funnel and filter paper (Whatman #4), or equivalent
- (k) Glass beads
- (l) Watch glass - about 80 mm diameter
- (m) Ion exchange column - 19 mm × 12 inches fitted with a coarse porosity scintered glass frit and teflon stopcock (Ace #5889-T), or equivalent

6.010C Reagents

(a) 0.0200M EDTA (Disodium dihydrogen ethylene-diamine tetraacetic acid dihydrate—Dissolve 7.44 g $\text{Na}_2\text{H}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (99 + % purity) in H_2O in 1 liter vol. flask, dilute to volume with H_2O and mix. Weigh accurately, 3 separate amounts of ACS primary standard CaCO_3 (about 80 mg) to give about 40 ml titration with 0.02M EDTA and transfer to 3 separate 500 ml Erlenmeyer flasks. To each, add 50 ml H_2O and enough HCl (1 + 3) to dissolve the CaCO_3 . Dilute each to about 150 ml with H_2O and add 15 ml 1N NaOH disregarding any ppt or turbidity. Add about 200 mg hydroxy naphthol blue indicator and titrate EDTA solution from pink to deep blue end point, using magnetic stirrer. Add last few ml EDTA solution dropwise. Molarity EDTA solution = $\frac{\text{mg CaCO}_3}{\text{ml EDTA} \times 100.09}$. Use the average molarity value of the three determinations.

- (b) Hydroxy Naphthol Blue Indicator (Mallinckrodt 5630)

(c) Potassium hydroxide—cyanide solution: Dissolve 280g KOH in 500 ml water. Cool, add 66g KCN, dissolve and dilute to 1 liter. (Use care in handling KCN, as HCN is formed upon contact with water and acids)

(d) 0.02M CaCO_3 —(ACS Primary Standard). Weight 2.000g CaCO_3 (dried at 100°C for 2 hrs) into 1 liter vol. flask. Add 500 ml distilled water and 10–12 ml 1 + 1 HCl. Heat just to boiling to dissolve. Dilute to volume with distilled water. Determine the relative strength ratio of the standardized EDTA solution to the CaCO_3 solution just before titrating samples (Reagent 6.009C (a) as follows: Pipet 3 separate 25.00 ml portions of EDTA solution into separate 250 ml vol. flasks. Dilute each to about 100 ml with H_2O and add 15 ml 1N NaOH, disregarding any ppt or turbidity. Add about 200 mg hydroxy naphthol blue indicator and titrate with the CaCO_3 solution from deep blue to pink end point, using magnetic stirrer. Add last few ml CaCO_3 solution dropwise.

$$\text{EDTA to CaCO}_3 \text{ ratio} = \frac{25.00 \text{ ml EDTA}}{\text{ml CaCO}_3 \text{ titrated}}$$

Use the average ratio from the three titrations.

(e) HCL (1 + 1) and (1 + 3)

(f) NaOH (1 + 5) and 1N

The following reagents are required only if phosphates are present:

(g) Amberlite IR-45 resin (Mallinckrodt 3328)

(h) 5% (w/v) Sodium Carbonate

(i) HCL (3 + 22)

(j) 10% KOH (w/v in water)

(k) Phenolphthalein indicator: 1% (w/v) in ethanol

6.010D Determination

(a) Weight 10g sample into 300 ml tall form beaker.

(b) Add 30 ml 1 + 1 HCl, several glass beads, cover with watch glass and place on hot plate in a fume hood.

(c) Slowly bring to a boil and digest for about 20 minutes.

(d) Cool, filter into 200 ml volumetric flask. Wash filter paper with water until 200 ml filtrate is obtained, stopper and mix.

At this point if phosphates are present, proceed with removal outlined in Section 6.010E.

(e) Pipet 20 ml aliquot into 400 ml beaker, add about 50 ml waer. (Use 10 ml aliquot for samples containing greater than 0.85 percent calcium.)

(f) On a magnetic stirrer in a fume hood add 200-300 mg hydroxy naphthol blue indicator (amount may vary from bottle to bottle), and adjust the pH to 12.5 ± 0.2 with KOH-KCN solution. (If pH exceeds 12.7, go back to step (e), as $\text{Ca}(\text{OH})_2$ will be precipitated and it is insoluble).

(g) Add 10-25 ml 0.02M EDTA (Amount depends on amount of calcium present. Must be in excess by at least 3 ml. Color should be green). Mix on magnetic stirrer.

(h) Titrate with 0.02M CaCO_3 to a permanent purple end point.

6.010E Removal of Phosphates (Required only if product has been dipped, soaked, or injected with or in phosphate solutions).

- (a) Initial and regeneration of resin - Mix. in a beaker, approximately 35g Amberlite IR-4B resin with three 250 ml portions of 5% sodium carbonate.
- (b) Wash with distilled water until washings indicate by phenolphthalein the absence of base.
- (c) Treat resin with three 250 ml portions of HCl (3 + 22), mixing thoroughly after each treatment.
- (d) Rinse with water until color is removed; transfer to column (apparatus M) with water. (The column is ready for use after water has drained to top of resin. The exchange capacity for phosphate is about 1,500 mg so a number of aliquots can be passed through the column before regeneration is necessary. Rinse column with about 250 ml water of until elute is colorless, before each use).
- (e) Transfer exactly 100 ml of sample solution from step 6.010D (d) above to a beaker. Adjust pH to 3.5 with 10% KOH, added drop by drop, using a pH meter and a magnetic stirrer.
- (f) Pass entire solution through the resin column, at a rate of 2-3ml/min., into a 250 ml volumetric flask.
- (g) Wash the beaker and column into the volumetric flask by passing through two 50 ml portions of water, the first at 2-3 ml/min., the second at 6-7 ml/min. Finally, freely pass enough water through column to get 250 ml total eluate. Stopper and mix.
- (h) Pipet 50 ml aliquot into 400 ml beaker and proceed as in steps 6.010D (f-h).

Note: If 10 ml aliquot was used, multiply by 2 to obtain percent calcium content.

6.010F Calculations

$$\% \text{ Calcium content} = C = [A - (B \times R)](0.08) \left(\frac{M}{0.0200} \right)$$

$$\% \text{ Bone for poultry} = [C - 0.015] F$$

where A = ml 0.02M EDTA

B = ml 0.02M CaCO_3

0.015 = correction for natural calcium in poultry tissue

F = 6.25 for young chickens

= 4.55 for turkeys and mature chickens

R = EDTA. to CaCO_3 Ratio

M = molarity of EDTA

When analyzing mechanically deboned poultry that includes product from different age groups, calculate the bone multiplier as follows: (% of young chicken) (6.25) + (% of mature chicken) (4.55) equals composite multiplier.

Bone content of "conventionally" cooked poultry

$$= \frac{[(C) - 0.015] (F)}{1.4}$$

If inspector designates % solids processed other than by "conventional" cooking methods, bone content of such products

$$= \frac{[(C) - 0.015] (F) (23)}{\% \text{ Solids}}$$

For nutritional analyses:

United States Recommended Daily Allowance (USRDA) = 1.0g

$$\text{mg/serving} = \frac{[A - (B \times R)](0.8\text{mg}) \left(\frac{M}{0.0200} \right) (f)}{W_t}$$

where A = ml 0.02M EDTA

B = ml 0.02M CaCO_3

f = serving size converted to appropriate dimensions (i.e., grams to ounces, etc.)

W_t = wt of sample in aliquot taken

Note:

%NFDm, in meat food products not containing mechanically separated (species) (MS(S)), by calcium method

$$= \frac{[(C) (1.3992)] - 0.024}{0.0184}$$

References

Wilson and Co. Method WC-29R1, 11/17/64

JAOAC, 49, 287 (1966)

JAOAC, 50, 195, 219 (1967)

Hart and Fisher, "Modern Food Analysis," Springer-Verlag, N.Y. (1971).

6.011 Amino Acid Analysis of Mechanically Separated (Species)

Nutritional value of any food product is derived from its content of protein, fat, and carbohydrate. The nutritional value of the contained proteins is based on the bioavailability and content of certain amino acids which are essential to nutrition. Current regulations list these essential amino acids as: isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Tryptophan is considered to be an essential amino acid by most nutritionists, but the current regulation does not list tryptophan because of the added cost of assay charged for its analysis by commercial laboratories. Required analysis of cystine/cysteine has also been deleted from analysis requirements because of economic reasons and sample turnover times.

However, if proper hydrolytic conditions are used, tryptophan content in a protein may be accurately determined with only a moderate increase in assay cost.

In order to ensure as complete recovery as possible of all amino acids, hydrolysis must be carried out under controlled conditions in the absence of oxygen. Thioglycolic acid, a reducing agent, is added to protect tryptophan and sulfur amino acids from oxidative destruction during hydrolysis. Norleucine acts as an internal standard to monitor sample handling losses.

Because mechanically separated (species) is made by crushing and grinding of marrow-containing bones, the nutritional value of the product can be diluted by the inclusion of excessive quantities of cartilaginous materials. Several amino acids which are present in collagen serve as indicators of collagen content. The most notable of these are hydroxylysine, hydroxyproline, proline, glycine, and alanine. Methionine content is often very low; cystine and tryptophan are usually absent.

The elution program developed for the amino acid analyzer separates all of the common amino acids plus hydroxyproline, hydroxylysine, norleucine, methionine sulfoxide and cysteic acid in a single run of 105 minutes. Traces of thioglycolic acid remaining in the hydrolysate do not interfere since thioglycolic acid elutes from the column well ahead of any amino acid.

Although tryptophan content in these products is very low, the Mark II computer system now in use on the Durrum D-500 analyzer gives adequate quantitation.

Interferences from fat and variations from varying moisture content of samples have been obviated by the preparation of acetone-chloroform powders of samples for analysis.

Instrument operator must be totally familiarized with operation and routine maintenance of the Durrum D-500 amino acid analyzer before beginning sample analysis. Each set of samples run should include random standards to check daily machine function.

The method below can also be used to determine the amino acid content of mechanically separated product made from poultry.

Small variations in elution time of each peak (± 15 sec) routinely occur, therefore the names, elution times and areas stored in computer memory on which quantitation of peaks is based should be the means from a ten-standard run. Also, because elution position and peak area are extremely pH sensitive, each new batch of buffer or ninhydrin must be checked for its affect on standard values. A new set of peak areas and elution times must be run for each new batch of reagents.

In order to maintain quality assurance of assay, coefficients of variation of the means of the standard series placed in computer memory must be kept as low as possible, preferably no greater than three (3.0).

6.011A Reagents:

- (a) Amino acid calibration standard (Pierce Chemical, Amino Acid Standard H, #20088)
- (b) Amino acids — purchase amino acid kit from Pierce, Regis, or other supplier

(c) Amino acids — hydroxyproline, hydroxylysine, tryptophan, norleucine, methionine sulfoxide and cysteic acid — purchase separately if not in kit.

(d) Thioglycolic and (Mercaptoacetic acid) — 98 + % pure

(e) Thiodiglycol (2,2 thiodiethanol)

(f) Hydrochloric acid, ACS reagent

(g) Na Citrate, ACS reagent

(h) Liquified phenol, reagent, MCB

(i) Pentachlorophenol preservative, Pierce

(j) Distilled water used in making buffers must be of high purity. Specific resistivity of at least four (4) megohms is recommended.

(k) 0.2N Na Citrate buffer, pH 2.2 (sample and standard diluent)

(l) 0.2N Na Citrate buffer, pH 3.00 ± 0.01

(m) 0.2N Na Citrate buffer, pH 3.25 ± 0.01

(n) 0.2N Na Citrate buffer, pH 4.25 ± 0.01

(o) 1.1N Na Citrate buffer, pH 7.90 ± 0.01

(p) Methyl cellusolve (Ethylene glycol monomethyl ether) for use in cold trap

(q) Supply of dry ice. Dry ice used in cold trap during evacuation of samples prior to hydrolysis and for removal of HCl and water following hydrolysis.

(r) Acetone glass-distilled (Burdick & Jackson)

(s) Chloroform, distilled-in-glass (Burdick & Jackson)

(t) HCl, 6N (1 + 1).

(u) Acetone:chloroform (3:1)

6.011B Equipment:

(a) Durrum D-500 Amino Acid Analyzer and associated equipment, or equivalent

(b) Small table top vise (clamp on type) for mounting filling jig for sample holding units (SHU's).

(c) Meat grinder, commercial model, Hobart or equivalent

(d) Plastic bags

(e) Balance, readable to 0.01 g, digital electronic type, 200-400 g capacity. Fisher #A-200 or equivalent.

(f) Polypropylene test tubes, 25 mm ID with fitted or screw tops (Falcon #2070) 50 ml capacity

- (g) Tissumizer, Tekmar Model SDT or equivalent
- (h) 1 ml volumetric pipettes, Class A
- (i) Vortex mixer or equivalent
- (j) Disposable Pasteur pipettes, 9" length and rubber bulbs
- (k) Hydrolysis tubes, two-piece, with vacuum fitting. (Kontes #K-896850) 18 mm × 140 mm, 25ml capacity
- (l) Heating block, multiple type, Lab-line or equivalent, 2 needed
- (m) Thermometer, 0 to 150° C
- (n) Vacuum pump, any make or model capable of at least 1.0u final vacuum and capacity of at least 55 l/min free air
- (o) McLeod gauge
- (p) Heavy wall vacuum tubing 1/4" ID
- (q) Buchler Evapomix and adapters
- (r) Infra-red heat lamp
- (s) Cold trap (Kontes #457500)
- (t) 18 × 150 mm Pyrex test tubes (Kimble #45048 or equivalent)
- (u) Test tube racks
- (v) Wash bottle
- (w) Dispensing pipette, Brinkman, 10 ml adjustable
- (x) Polypropylene snap-top disposable test tubes, 17 × 100 mm (Curtin Matheson Scientific #252-981)
- (y) Disposable microfilters, Gelman Acrodisc or Millipore; Gelman Acrodisc AN-450 with pre-filter, Gelman Instruments, Ann Arbor, MI or Millipore Millex SLHA 0250S, Millipore, Bedford, MA
- (z) 10 ml syringes, plastic disposable with luer fitting
- (aa) pH meter, digital, readable to ± 0.01 pH unit
- (bb) Labeling tape
- (cc) Marker pens
- (dd) Rubber tubing 1/4" ID
- (ee) Microliter syringe, 100 or 250 ul Hamilton or Glenco
- (ff) Parafilm
- (gg) Heavy duty ring stand

- (hh) Analytical balance
- (ii) Buchner funnel, 60 ml, with coarse porosity fritted disk
- (jj) Suction flask and vacuum adapters
- (kk) Statistical calculator with capacity for calculation of std. deviation, coefficients of variance, linear regression, etc. (Texas Instruments SR51-II or equivalent)

6.011C Determination:

- (a) Grind samples for analysis at least 2X by passage through a meat grinder fitted with a plate having 1/8" holes.
- (b) Weigh 2-3 g sample into 25 mm diameter tube.
- (c) Add 20 ml acetone:chloroform from dispensing pipette.
- (d) Homogenize 1 min with tissumizer.
- (e) Pour blended sample into 60 ml Buchner funnel having a coarse porosity frit.
- (f) Add second 20 ml portion of acetone:chloroform to sample tube and run blender again for a few seconds to clean blade. Add washings to Buchner funnel.
- (g) Apply vacuum and remove acetone. Continue vacuum until sample is air-dry.
- (h) When sample is dry (ca 2-4 min) remove from funnel and place in labeled disposable polypropylene tube.
- (i) Weigh duplicate samples, 7.0 ± 0.1 mg (use disposable plastic weighing pan). Transfer weighed sample to labeled hydrolysis tube. Wash any adhering particles of sample from weighing pan into tube by dropwise addition of (1 + 1), HCl (0.5 - 1.0 ml).
- (j) Add 1.0 ml norleucine internal standard (2.5 μ moles ml in pH 2.2 citrate buffer).
- (k) Add 100 μ l thioglycolic acid to hydrolysis tube.
- (l) Add 1.0 ml 12N (conc) HCl to each tube.
- (m) Assemble hydrolysis tubes, then fill cold trap with methyl cellulosedry ice mixture. Add dry ice in small pieces at first, waiting after each addition until violent frothing stops. Continue adding dry ice until trap is full. Do not turn on vacuum pump until trap is totally chilled. Check condensate reservoir and empty if any liquid is present. Rotate McLeod gauge so that mercury is in reservoir. Attach vacuum hose to first sample, open stopcock and turn on vacuum pump.
- (n) Evacuate tube. Agitate or shake tube gently during evacuation to aid in release of dissolved oxygen. If bubbles begin to rise up walls of tube, shut off vacuum momentarily by closing stopcock of hydrolysis tube until bubbles break. Continue evacuating tubes until internal pressure drops to 50-100u. Close stopcock and remove vacuum hose.
- (o) Attach hose to next sample tube, open stopcock and evacuate as above. If pump oil is not contaminated and no leaks in tube parts or vacuum hose and connections are present, evacuation of each tube should require only three minutes.
- (p) After tubes are evacuated, place in heating block at $110 \pm 1^\circ\text{C}$ and hydrolyze for 24 hours.
- (q) After 24 hours, remove tubes from heating block, and cool to room temperature.

- (r) Open stopcock and release vacuum.
- (s) Use 9" disposable Pasteur pipette and transfer hydrolysate to clean labeled 18 × 150 mm pyrex tube.
- (t) Rinse hydrolysis tube with several small rinses of distilled water, adding each rinse to 18 × 150 mm tube.
- (u) Attach Evapomix adapter to top of tube and mount tube in Evapomix. Be sure cold trap is chilled before turning on vacuum. Flow cold water through Evapomix condenser. Adjust initial bath temperature of 40°C. Turn motor of Evapomix on and rotate tubes to spin liquid up walls of tube. Turn on vacuum pump, then open stopcock and begin evaporating liquid. Gradually increase bath temperature to 60°C. Keep cold trap filled with dry ice during evaporation to prevent vapors from entering vacuum pump and contaminating oil.
- (v) Evaporate samples to dryness, then remove each sample from Evapomix and wash down walls of tube with small amount of distilled water.
- (w) Remount Evapomix adapter on tube and again evaporate to dryness (or as near dryness as possible, thioglycolic acid does not evaporate readily).
- (x) When evaporation is complete, remove samples from Evapomix and add 5.0 ml pH 2.2 sample dilution buffer to each tube. Cover top of tube with square of Parafilm.
- (y) Mix sample well on vortex mixer. Be sure small reddish-brown button of thioglycollate disappears from bottom of tube.
- (z) Remove plunger from disposable syringe and attach disposable microfilter to end of barrel. Pour sample into syringe barrel, replace plunger and filter sample into labeled snap-top disposable polypropylene tube.
- (aa) Load 20 ul of each diluted sample into cartridge (SHU) for analysis of common amino acids.

6.011D Calculations:

- (a) Add up totals of essential amino acids and total amino acids.
- (b) Calculate essential amino acids, total amino acids ratio, i.e., essential amino acids = total of isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Divide total of above values (ng, mg, etc.) by total value (ng, mg, etc.) of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, alanine, arginine, aspartic acid, histidine, hydroxyproline, glutamic acid, glycine, proline, serine, and tyrosine, and multiply by 100. Do not include norleucine and other minor constituent amino acids in calculations. Express essential amino acid content as percent value.
- (c) All sample hydrolysates used to calculate essential amino acid content must have internal standard recovery of greater than 90 percent.
- (d) Average values of duplicate hydrolysates and report final answer as average of duplicate essential amino acid content measurements. Samples used for duplicate averaging must not differ from each other by more than 10 percent.

6.011(LC) Amino Acid Analysis of Mechanically Separated (Species) (HPLC Method)

Nutritional value of any food product is derived from its content of protein, fat, and carbohydrate. The nutritional value of the contained proteins is based on the bioavailability and content of certain amino acids which are essential to nutrition. Current regulations list these essential amino acids as: isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Tryptophan is considered to be an essential amino acid by most nutritionists, but the current regulation does not list tryptophan because of the added cost of assay charged for its analysis by commercial laboratories. Analysis of the non-essential amino acid cystine is also not required by current regulations and is not included in this procedure.

If proper hydrolytic conditions are used however, tryptophan content in a protein may be accurately determined with only a moderate increase in assay cost.

The method below can also be used to determine the amino acid content of mechanically separated product made from poultry.

In order to ensure as complete recovery as possible of all amino acids, hydrolysis must be carried out under controlled conditions in the absence of oxygen. Thioglycolic acid, a strong reducing agent, is added to protect tryptophan and sulfur amino acids from oxidative destruction during hydrolysis. Norleucine acts as an internal standard to monitor sample handling losses.

Because mechanically separated species products are made by crushing and grinding of marrow containing bones, the nutritional value of the product can be diluted by the inclusion of excess quantities of cartilaginous materials. Several amino acids which are present in collagen serve as indicators of collagen content. The most notable of these are hydroxyproline, proline, glycine, and alanine. Methionine content is often very low; cystine and tryptophan are usually absent.

The elution program developed for the high pressure liquid chromatographic separation of amino acids will also successfully separate hydroxyproline, proline, norleucine, methionine sulfoxide, and cysteic acid and all common amino acids in a single run of 90 minutes. Traces of thioglycolic acid remaining in the hydrolysate do not interfere since thioglycolic acid elutes from the column well before any of the amino acids. Although tryptophan content of these products is very low, adequate quantitation may be achieved by modification of the elution program (See 6.011LC Appendix C.) Interferences from fat and variations from moisture content have been obviated by the preparation of acetone-chloroform powders for analysis.

The instrument operator must be totally familiar with the operation and routine maintenance of the Waters Associates amino acid analysis system before beginning sample analysis.

The Waters HPLC Amino Acid Analysis System is an automated gradient liquid chromatograph tailored for the analysis of free amino acids. The amino acid separation is accomplished via the use of a combined pH — ionic strength gradient on a strong cation exchange column; the amino acids are derivatized with orthophthalaldehyde (OPA) following elution from the column and detected by fluorescence. The secondary amino acids proline and hydroxyproline, are oxidized with hypochlorite prior to reaction with OPA.

6.011(LC)A Reagents

- (a) Amino acid calibration standard (Pierce Chemical, Amino Acid Standard H, #20088).
- (b) Amino acids — purchase amino acid kit from Pierce, Regis, or other supplier.
- (c) Amino acids — hydroxyproline, hydroxylysine, tryptophan, norleucine, methionine sulfoxide and cysteic acid — purchase separately if not included in kit.
- (d) Thioglycolic acid (Mercaptoacetic acid) 98+ % pure.

- (e) o-phthalaldehyde (OPA) Alfa Products (16344), or equivalent.
- (f) Hydrochloric acid — Baker Ultrex grade.
- (g) Sodium citrate, ACS Reagent grade — Baker 1-3646, or equivalent.
- (h) Brij 35-30% solution — Fisher CS-285-3, or equivalent.
- (i) Distilled water used in making buffers must be of high purity — minimum specific resistivity of at least ten megohms is required.
- (j) Potassium hydroxide ACS — Baker 3140.
- (k) Sodium chloride ACS Reagent — Fisher S-671, or equivalent.
- (l) Sodium hydroxide ACS Reagent — Fisher S-318, or equivalent.
- (m) Methyl Cellusolve (Ethylene glycol monomethyl ether), for cold trap.
- (n) Dry ice for cold trap.
- (o) Acetone, distilled in glass — Burdick and Jackson, or equivalent.
- (p) Chloroform, distilled in glass — Burdick and Jackson, or equivalent.
- (q) Acetone: Chloroform (3:1).
- (r) Boric acid granular — Baker (00945).
- (s) 3-Mercaptopropionic acid — Sigma (M 6750), or equivalent.
- (t) Methanol — Burdick and Jackson, or equivalent.
- (u) 2-propanol — Burdick and Jackson, or equivalent.
- (v) Sodium hypochlorite solution, 5% — Aldrich Chemicals 23,930-5, or equivalent.

6.011(LC)B. Equipment:

- (a) Waters Associates Amino Acid Analysis System — Waters Associates, Milford, MA.
- (b) Meat grinder, commercial model. Hobart, or equivalent.
- (c) Plastic bags for sample storage.
- (d) Balance, Mettler, Model K7T, readable ± 0.1 g, or equivalent.
- (e) Polypropylene test tubes, 25 mm i.d. with fitted or screw tops — Falcon #2070, 50 ml capacity, or equivalent.
- (f) Tissumizer, Tekmar Model SDT, or equivalent.
- (g) 1 ml volumetric pipets, class A.
- (h) Vortex mixer, or equivalent.

- (i) Disposable Pasteur pipettes, 9 in. length, and rubber pipet bulbs.
- (j) Hydrolysis tubes, two piece with vacuum fitting — Kontes #K-896950, 18 mm × 140 mm, 25 ml capacity.
- (k) Heating block, multiple type, Lab-Line, or equivalent (need 2), with insert blocks having 18 mm holes.
- (l) Thermometer, 0° to 150°C.
- (m) Vacuum pump, direct drive, double stage (CSC Scientific 90703-001), or equivalent.
- (n) McLeod gauge.
- (o) Heavy wall vacuum tubing 1/4" i.d.
- (p) Buchler Evapomix and adapters.
- (q) Infra-red heat lamp.
- (r) Cold trap — Kontes #457500.
- (s) 18 × 150 mm Pyrex test tubes — Kimble #45048, or equivalent.
- (t) Test tube racks.
- (u) Wash bottle.
- (v) Dispensing pipette — Brinkman, 10 ml adjustable, or equivalent.
- (w) Polypropylene snap-top disposable test tubes, 17 × 100 mm — Curtin Matheson #252-981, or equivalent.
- (x) Disposable microfilters, Gelman Acrodisc or Millipore: Gelman Acrodisc AN-450 with pre filter, Gelman, Instruments, Ann Arbor, MI or Millipore Millex SLHA 0250S, Millipore, Bedford, MA.
- (y) 10 ml syringes, plastic disposable, with luer fitting.
- (z) pH meter, digital, Corning Model 125, or equivalent.
- (aa) Labeling tape.
- (bb) Marker pens.
- (cc) Microliter, syringe, 100 ul.
- (dd) Parafilm.
- (ee) Heavy duty ring stand.
- (ff) Analytical balance, Mettler, Model H54AR, or equivalent.
- (gg) Buchner funnel, 60 ml, with coarse porosity fritted disk.
- (hh) Suction flask and vacuum adapters.

6.011(LC)C. Standards

(a) Amino acid standard is prepared by mixing 1 ml of calibration standard H, 1 ml of 2.5 μ M/ml hydroxyproline and 1 ml of 2.5 μ M/ml norleucine. The mixture is made up to a final volume of 10 ml using buffer A. Store the standard in the refrigerator and prepare fresh every 2 weeks. Note that with this dilution and a 20 μ l injection the column load is 5.0 nanomoles per amino acid.

(b) For addition of hydroxyproline and norleucine to the mixed amino acid standard dissolve each in 0.1 N HCl solution at 2.5 μ M/ml (store in refrigerator). Other amino acids listed under Reagents, item (c) may be added to the mixed amino acid standard for peak identification purposes only. If tryptophan is to be added, it must be dissolved in a neutral or basic solution such as the "B" buffer, since it is degraded in acidic solution. A special elution program is required for tryptophan quantitation.

6.011(LC)D. Composition of Elution Buffers and Post Column Solutions. All Formulations are for 1-liter Quantities.

(a) Buffer A; 0.2N Na^+ , 2% 2- propanol, pH 2.95.

- | | |
|---|--------|
| (1) Sodium citrate dihydrate | 19.6 g |
| (2) 2-propanol | 20.0 g |
| (3) Hydrochloric Acid (Ultrex) to pH 2.95 | |

(b) Buffer B; 1.1 N Na^+ , pH 7.1-7.4

- | | |
|--|--------|
| (1) Sodium citrate dihydrate | 19.6 g |
| (2) Sodium chloride | 52.5 g |
| (3) Hydrochloric acid (Ultrex) to pH 7.1-7.4 | |

(c) Post Column Reaction Buffer; 0.5M Boric Acid — KOH, pH 10.2-10.7

- | | |
|-------------------------|--------|
| (1) Boric Acid | 30.5 g |
| (2) Potassium Hydroxide | 26.3 g |

(d) OPA Solution

- | | |
|-----------------------------------|-----------|
| (1) Orthophthaldehyde | 700 mg |
| (2) Methanol | 10 ml |
| (3) 3-mercaptopropionic acid | 2.0 ml |
| (4) BRIJ 35 | 1.0 ml |
| (5) 0.5 M Boric Acid — KOH buffer | 1.0 liter |

NOTE: Dissolve the OPA and 3-mercaptopropionic acid in the methanol and then combine with Boric acid — KOH BRIJ 35 solution.

(e) Hypochlorite Solution

- | | |
|-----------------------------------|-----------|
| (1) Sodium Hypochlorite | 2.0 ml |
| (2) BRIJ 35 | 1.0 ml |
| (3) 0.5 M Boric acid — KOH buffer | 1.0 liter |

NOTE: Degas K Borate — BRIJ 35 mixture thoroughly before adding sodium hypochlorite.

6.011(LC)E. Preparation of Buffers and Solutions

- (a) Dissolve salts in purified water at 90 percent of final volume.
- (b) Adjust pH with hydrochloric acid.
- (c) Make up to final volume in volumetric flask using purified water.
- (d) Filter solution through 0.45 μ aqueous compatible filter.
- (e) Transfer eluent to amber bottles and purge for 15 minutes with nitrogen.
 - (1) Buffer A and B may be made up in 4 liter quantities and stored two weeks in the refrigerator.
 - (2) Post column reaction buffer may be prepared in 4 liter quantities and is stable at room temperature.
 - (3) The OPA and hypochlorite solutions must be stored in an inert atmosphere and prepared fresh every 48 hours.

6.011(LC)F. Sample Preparation

- (a) Grind samples for analysis at least two times by passage through a meat grinder fitted with a plate having $\frac{5}{64}$ " holes. (For atypical samples containing large amounts of skin and or bone, blend in a commercial blender after pre-chilling both blender and samples with liquid nitrogen.
- (b) Weigh 2-3 g sample into 25 mm diameter tube.
- (c) Add 20 ml acetone: chloroform from dispensing pipette.
- (d) Homogenize 1 min. with tissumizer.
- (e) Pour blended sample into 60 ml Buchner funnel.
- (f) Add second 20 ml portion of acetone:chloroform to sample tube and blend for a few seconds to clean blade. Add washings to Buchner funnel.
- (g) Apply vacuum and filter off acetone:chloroform solvent. Continue vacuum until sample is air-dry. If after drying sample has sticky texture, blend again with solvent and air-dry. A dry, free-flowing powder must be produced.
- (h) When sample is dry (ca 2-4 min.) transfer to a clean, labeled disposable polypropylene tube.
- (i) Weigh duplicate samples 10.0 ± 0.1 mg (use disposable plastic weighing pan). Transfer weighed sample to labeled hydrolysis tube. Wash any adhering particles of sample from weighing pan into tube by dropwise addition of (1 + 1), HCl (0.5-1.0 ml).

(j) Add 1.0 ml norleucine internal standard (2.5 μ moles/ml in 0.1 N HCl).

(k) Add 150 μ l thioglycolic acid to hydrolysis tube.

(l) Add 1.5 ml 12 N (conc) HCl to each tube, followed by 0.5 ml deionized water.

(m) Assemble hydrolysis tubes, then fill cold trap with methyl cellulose and dry ice mixture. Add dry ice in small pieces at first, waiting after each addition until violent frothing stops. Continue adding dry ice until trap is full. Do not turn on vacuum pump until trap is totally chilled. Check condensate reservoir and empty if any liquid is present. Rotate McLeod gauge so that mercury is in reservoir. Attach vacuum hose to first sample, open stopcock and turn on vacuum pump.

(n) Immerse tubes in ice bath for first 2-3 min. of evacuation period. Agitate or shake tube gently during evacuation to aid in release of dissolved oxygen. If bubbles begin to rise up walls of tube, shut off vacuum momentarily by closing stopcock of hydrolysis tube until bubbles break. Continue evacuating tubes until internal pressure drops to 50-100 μ . Close stopcock and remove hose.

(o) Attach hose to next sample tube, open stopcock and evacuate as above. If pump oil is not contaminated and no leaks in tube parts or vacuum hose and connections are present, evacuation of each tube should be complete in 2-4 min.

(p) After tubes are evacuated, place in heating block at 145° and hydrolyze for 4 hrs. Tubes should be protected from air drafts during hydrolysis period to prevent cooling of upper part of tube. A convenient cover can be fashioned by covering a wire mesh test tube basket with aluminum foil. Temperature in block must be maintained at a minimum of 145°C, but must not exceed 150°C during hydrolysis period.

(q) After 4 hours, remove tubes from heating block, and cool to room temperature.

(r) Open stopcock and release vacuum.

(s) Use 9" Pasteur pipette and transfer hydrolysate to clean labeled 18 × 150 mm pyrex tube.

(t) Rinse hydrolysis tube with small rinses of distilled water, adding each rinse to 18 × 150 mm tube. Restrict volume used for rinsing to not more than 1.0 ml.

(u) Attach Evapomix adapter to top of tube and mount tube in Evapomix. Be sure cold trap is chilled before turning on vacuum. Flow cold water through Evapomix condenser. Adjust initial bath temperature to 60°C. Turn motor of Evapomix on and rotate tubes to spin liquid up walls of tube. Turn on vacuum pump, then open stopcock gradually to begin evaporating liquid. Total volume of hydrolysate plus rinses in evaporator tube should not exceed 5 ml to prevent sample losses from bumping during early stages of evaporation. Keep cold trap filled with dry ice during evaporation to prevent vapors from entering vacuum system, and contaminating pump oil. Turn on infrared heat lamp to assist in driving vapor out of vacuum adapter bulbs.

(v) Evaporate samples to near dryness, then remove each sample tube from Evapomix and wash down walls of tube with small amount of distilled water.

(w) Remount Evapomix adapter on tube and again evaporator to dryness. (Note: thioglycolic acid does not evaporate completely). Sharp acid odor of HCl should be absent from hydrolysates at end of evaporation.

(x) When evaporation is complete, remove samples from Evapomix and add 10.0 ml of "A" buffer to each tube. Cover top of tube with square of Parafilm.

(y) Mix sample well on vortex mixer. Be sure reddish-brown button of thioglycolate disappears from bottom of tube.

(z) Remove plunger from disposable syringe and attach disposable microfilter to end of barrel. Pour sample into syringe barrel, replace plunger and filter sample into labeled snap-top disposable polypropylene tube. Store filtered hydrolysates in refrigerator. Stable one week. Dried undiluted hydrolysates may be stored in a freezer for an indefinite period.

6.011(LC)G. Daily HPLC Equipment Start-up

(a) Turn switches to the following indicated positions:

(1) Solvent delivery system, pumps A and B; switch toggle switch "ON" with flow control set at 0.0 ml/min on digital meter.

(2) Post column reaction pumps; switch toggle switch on each pump ON and set unit switch to auto.

(3) Temperature control module: press power button ON and set temperature to 60°C. Adjust control as needed.

(4) WISP: Press power button ON.

(5) Data module: Press power button ON.

(6) System controller: Press power button ON.

(7) M-420 Fluorometer: Press power button ON. Set attenuator at 2. Turn SPAN completely clockwise.

(b) Program loading

(1) Insert tape into system controller.

(2) After the Water's logo appears, press NEXT MOD to access the system monitor page.

(3) Type date as a 6 digit parameter, press NEXT VAL, type time as a 6 digit parameter, press NEXT VAL, type an operator code and press ENTER.

(4) Press NEXT PAGE 4 times to access the tape operation page.

(5) Type "load", press ENTER.

(6) Type "AAA.200" press ENTER. Program loading is complete when "to continue press enter" appears on the screen. At this point all system set points have been automatically entered by the tape. See appendix A for a detailed listing of parameters.

(c) Pump Priming and Purging

(1) Place inlet lines in appropriate buffer bottles positioned at a level above the pumps.

(2) Press ENTER, press NEXT MOD to access the pump monitor page.

(3) Press NEXT PAGE to access initial condition page.

(4) Open relief valve on pump "A".

(5) STOP! Is the relief valve open? Type a flow of 10 ml/min. 100 percent A and press ENTER. Prime the M6000 A with buffer A using a 10 ml syringe on the draw off valve. A steady stream of effluent should be seen from the relief valve tube.

(6) Type 0 percent A, 100 percent B and press ENTER. Prime the B pump with buffer B.

(7) Type a flow of 0 ml/min. 100 percent A. 0 percent B and press ENTER.

(8) Press NEXT PAGE until pump monitor page is accessed.

(9) STOP! Is the flow 0? If no flow is present, close relief valve on pump A.

(10) Prime the post column reaction pumps with appropriate solutions by drawing 20 ml of solution through the pump using a syringe attached to the draw off valve.

(d) Column and System Equilibration

(1) After the column has reached 60°C, access the pump monitor page and press ENTER.

(2) Type 2 for editing pump set, type 2 for running pump set and press ENTER. The system is now operating at a flow of 0.5 ml/min of 100 percent buffer A. The pressure should stabilize at 700 to 1200 psi.

(3) Allow system to equilibrate for 30 min.

6.011(LC)H. Calibration Table and Standard Plan

(a) Place calibration standard in position 1 of WISP.

(b) Press RUN/STOP button on WISP and allow 90 minutes for standard run.

(c) Using the above standard run prepare a calibration table as illustrated in Table 1.

(d) Enter the values of the calibration table into the data module.

(1) Press CLEAR, ENTER.

(2) Press 10, ENTER.

(3) Press 1, ENTER. This erases any previous calibration tables.

(4) Press NEXT (ID). Enter ID number of first amino acid from table.

(5) Press NEXT (RT). Enter retention time of first amino acid.

(6) Press NEXT (CO). Enter concentration of first amino acid.

(7) Press NEXT, NEXT (ID). Enter ID number of second amino acid. Continue until table is complete.

(8) When last amino acid has been entered press ENTER three times.

(9) List and check calibration table by entering 99 ENTER, 3 ENTER.

(10) A wrong value may be removed by reentering the entire line as is, except enter 0 for concentration. This will remove the entire line. A line cannot be over written for correction. A new line may be added at any time.

(e) Enter the recalibration sequence into module. The recommended sequence is to average two standards every 14 samples.

(1) Enter a value of 0 for parameter 34.

(2) Enter a value of 1 for parameter 36.

(3) Enter a value of 2 for parameter 37.

(4) Enter a value of 14 parameter 38.

- (5) Enter a value of 0 for parameter 39.
- (6) Load the WISP tray such that standards are in position 1 and 15.
- (7) Calibration sequence can be re-initiated by entering the value for parameter 37.

6.011(LC)I. Analysis and Calculations

- (a) With samples and standards loaded in WISP initiate the analysis by pushing the RUN/STOP button on the WISP.
- (b) The total weight of essential amino acids will be reported as Group 1, nonessentials as Group 2, and percent norleucine in Group 3 at the bottom of each report.
- (c) Weight percent essential amino acids is calculated by dividing Group 1 value by the sum of Group 1 and Group 2 value.
- (d) Norleucine recovery value must be greater than 90 percent.
- (e) Report percent essential amino acids as average of duplicate assays. Duplicates may be averaged only if percent essential amino acids in samples differs by less than 10 percent.

6.011(LC)J. Instrument Shutdown

- (a) On a weekly basis flush the column with 0.1 N NaOH for 30 minutes at a flow of 0.5 ml/min.
- (b) During short periods of inactivity the equipment is programmed to reduce flow of buffer A and buffer B to a total flow of 0.2 ml/min. Post column pumps will automatically turn off.
- (c) For 2-4 day shutdown periods, flush column with 0.2N NaOH and turn off power to all modules.
- (d) For periods longer than a week refer to the operators manual.

6.011(LC) Table I
Calibration Worksheet

Amino Acid	ID Numbers	Retention Time	Amount ng injected
Hydroxyproline	1002	Enter values	655
Aspartic Acid	1002	"	665
Threonine	1001	"	595
Serine	1002	"	525
Glutamic Acid	1002	"	735
Proline	1002	"	575
Glycine	1002	"	375
Alanine	1002	"	446
Valine	1001	"	585
Methionine	1001	"	745
Isoleucine	1001	"	655
Leucine	1001	"	655
Norleucine	1003	"	100 *
Tyrosine	1002	"	905
Phenylalanine	1001	"	825
Lysine	1001	"	730
Histidine	1002	"	775
Arginine	1002	"	870

* The actual injected quantity is 655 ng; 100 is entered for this value to allow the report to contain the percent recovery for norleucine.

6.011(LC) APPENDIX A

System Controller and WISP Parameters

Pump Controller Conditions

Standby Conditions for run set 02

Pressure: 0000 Delay Time: 0
High Limit: 2500 Plot: %B

Pump	Solvent	Comp
A	Buf A	2.95
B	1.1 Na	7.10
C		0

Initial Conditions/Gradient Table

Time	Flow	%A	%B	%C	Curve
Initial	.50	100	0	0	*
45.00	.50	20	80	0	08
60.00	.50	0	100	0	08
80.00	.50	100	0	0	11
120.00	.20	50	50	0	11

External Events

No.	Description
1	Hypo
2	OPA

Time	No.	Status
10.00	1	On
10.10	2	On
85.00	1	Off
85.10	2	Off

Sample POS 00. Inj volume 0020. No of Inj 1*. Run Time 00.00. AM WISP codes generated.

* Note: Standard positions 1 and 15 are set at 2 injections/standard.

6.011(LC) APPENDIX B

Data Module Parameters

Code No.	Description	Symbol	Value
0	Date	dA	Auto set via system controller
1	Time of Day	ti	Auto set via system controller
2	Chart Speed	CS	0.25
3	Plot Mode	PL	0
4	Pen 2	P2	0
5	Pen 1 Zero	01	10
6	Pen 2 Zero	02	10
7	Auto Zero	Ao	1
8	LC/GPC	LC	1
9	Calib/Anal	CA	(Set via parameter 37)
10-14	Calibration parameters discussed in Calibration Table and Stand Run Section 6.010 (LC)G.		
20	Auto Parameter	AP	1
21	Peak Width	P—	20
22	Noise Rejection	Nr	250
23	Area Rejection	Ar	1000
33	Report Format	—	100 (for a short report 0)
45	Run number start		0
63	Report Format	—	1000 (this configuration is required to prepare types for short report 0).

6.011(LC) APPENDIX C

Modification for Inclusion of Tryptophan Analysis

Modify method steps as listed below only when tryptophan analysis is to be added to the methodology. Normal verification analyses do *not* require analysis of tryptophan.

6.011(LC)C Standards

(a) Amino acid standard is prepared by pipetting 1 ml of calibration standard H, 1 ml of 2.5 M/ml hydroxyproline, 1 ml of 2.5 M/ml norleucine and 1 ml of 0.625 M/ml tryptophan into a 10 ml volumetric flask. Make up to final volume using Buffer "A". Store the standard in the refrigerator. Make fresh weekly. Note that with this dilution and a 20 μ l injection, the column load is 1.25 nanomoles for tryptophan and 5.0 nanomoles for all other amino acids.

(b) For addition of tryptophan to the mixed amino acid standard dissolve tryptophan in buffer "B" at 0.625 M/ml. Store in refrigerator. Make fresh monthly.

6.011(LC)F Sample Preparation

(i) Weigh duplicate samples 10.0 ± 0.1 mg (use disposable plastic weighing pan). Transfer weighed sample to labeled hydrolysis tube. Wash any adhering particles of sample from weighing pan into tube by dropwise addition of (1 + 1), HCl (0.5 – 1.0 ml).

(k) Add 150 μ l thioglycolic acid to hydrolysis tube.

(l) Add 1.5 ml 12 N (conc) HCl to each tube, followed by 0.5 ml deionized water.

(p) After tubes are evacuated, place in heating block at 145°C and hydrolyze for 4 hours. Tubes should be protected from air drafts during hydrolysis period to prevent cooling of upper part of tube. A convenient cover can be fashioned by covering a wire mesh test tube basket with aluminum foil. Temperature in block must be maintained at a minimum of 145°C, but must not exceed 150°C during hydrolysis period.

(q) After 4 hours, remove tubes from heating block, and cool to room temperature.

(t) Restrict volume used for rinsing to not more than 1.0 ml.

(u) Initial water bath temperature may be raised to 60°C to speed evaporation. Total volume of hydrolysate plus rinses in evaporator tube should not exceed 5 ml to prevent sample losses from bumping during early stages of evaporation. Liquid must spin up walls of tube before vacuum is applied.

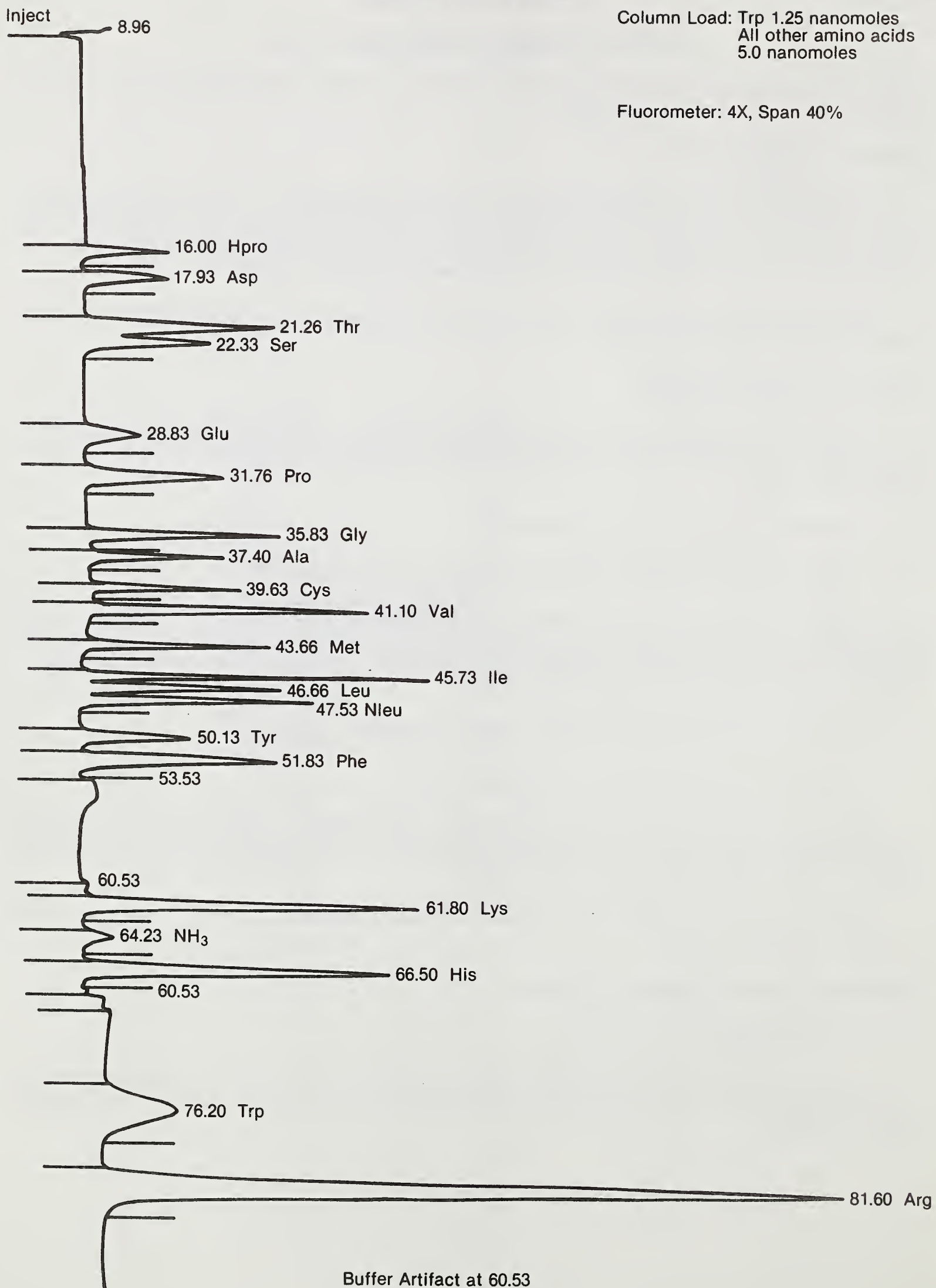
(x) When evaporation is complete, remove samples from Evapomix and add 10 ml of "A" buffer to each tube. Cover top of tube with square of parafilm.

6.011(LC)G Daily HPLC Equipment Start-up

(b) Program loading.

(c) Type "AAA.006" press ENTER. Program loading is complete when "to continue press enter" appears on the screen. At this point all system set points have been automatically entered by the tape. See Appendix A for a detailed listing of parameters.

APPENDIX C (continued)



6.011(LC)H Calibration Table and Standard Run

(e) (1) Enter a value of 1001 for parameter 33

(2) Enter a value of 0 for parameter 34

(3) Enter a value of 2 for parameter 36

(4) Enter a value of 2 for parameter 37

*(5) Enter a value of 14 for parameter 38

(6) Enter a value of 0 for parameter 39

(7) Enter a value of 68 for parameter 73

*(8) Load the WISP tray such that standards are in positions 1 & 15

(9) Calibration sequence can be re-initiated by entering the value of parameter 37.

* Note: Parameter 38 is the number of samples before re-calibration. If more frequent re-calibration is used, the values in (5) and (8) must be changed accordingly.

Table 1

Change high pressure limit to 1500. Add tryptophan to bottom of list of names of amino acids. Tryptophan ID number is 1001. Amount in ng is 255.

Modify external events table as follows:

<u>Time</u>	<u>Number</u>	<u>Status</u>
0.10	1	on
0.20	2	on
*68.00	1	off
110.00	2	off

Note that with this modification both tryptophan and arginine are quantitated with hypochlorite oxidizer off. The relative quantity of tryptophan in the standard is reduced for closer quantitation of the small amounts of tryptophan present in these sample types. Arginine appears as a very large peak because of absence of oxidizer, but is adequately quantitated. See enclosed standard chromatogram.

Appendix B

* Parameter 73 is one-half noise rejection. The 68.00 time noted above is the time of return to baseline following histidine elution for the particular column in use at CDLB. This time must be determined by the analyst for each column and the value in minutes entered in the external events table as above. The reduction to one-half of the noise rejection parameter facilitates better quantitation of the small quantities of tryptophan present in MS(S) samples.

6.012 Determination of Fluoride in Mechanically Separated (Species)

6012A. Theory

Mechanically Separated (Species) samples are defatted with petroleum ether. Disodium EDTA is added to complex calcium, and a total ionic strength adjustor is added to complex interfering ions such as aluminum and iron and to provide a constant background ionic strength, decomplex fluoride, and adjust solution pH. Final sample solution is then measured on a millivolt meter with fluoride-specific ion and reference electrodes.

6.012B. Apparatus

- (a) pH/mv meter — Orion Model 701, or equivalent.
- (b) Fluoride ion electrode — Orion No. 94-09, or equivalent.
- (c) Reference electrode — Single-junction, Orion No. 90-01, or equivalent.
- (d) Tissue blender — Model SDT Tissumizer, Tekmar Co., Cincinnati, OH 45222, or equivalent.
- (e) Centrafuge — Equipped with accessories for 250 ml centrafuge bottles (IEC PR-600, or equivalent).
- (f) Centrafuge bottles — 250 ml capacity, Corning No. 1280, or equivalent.
- (g) Silicone stoppers — No. 6 (Thomas No. 8747-E65, or equivalent).
- (h) Constant temperature water bath with cover — Blue M Model MW1120A, or equivalent.
- (i) Polyethylene bottles — 16 and 32 oz. Thomas No. 1704-C38 and 1704-C48, or equivalent.
- (j) Polypropylene beakers — 100 and 250 ml, Thomas No. 1541-F35 and 1541-F45, or equivalent.
- (k) Volumetric flasks — 11 and 100 ml, Thomas No. 4997-B70 and 4997-B47, or equivalent.

6.012C. Reagents

- (a) Petroleum ether — distilled in glass, Burdick and Jackson, Muskegon, MI 49442, or equivalent.
- (b) Disodium ethylenediamine tetraacetic acid — 5% w/v, in distilled water.
- (c) Total ionic strength adjustor (TISAB) — Dissolve 116 g NaCl and 0.6g sodium citrate in 114 ml glacial acetic acid and 1000 ml distilled water. Add 5N NaOH until pH is 5.0-5.5. Cool to room temperature and dilute to 2 liters with distilled water.
- (d) Standard solutions (Prepare in vol flasks and store in polyethylene bottles)
 - Stock Solution A. — Carefully weigh 2.2101g NaF and dilute to 1 liter with distilled water (1000 ppm F^-).
 - Stock Solution B. — Dilute 10 ml stock solution A to 1 liter with distilled water (10 ppm F^-).
 - Stock Solution C. — Dilute 10 ml stock solution B to 100 ml with distilled water (1.0 ppm F^-).
- (e) Working standards (in volumetric flasks):

(1) Fluoride ion concentration, (F^-) = 0.03 ppm. To 3 ml stock solution C, add 20 ml TISAB and dilute to 100 ml with distilled water.

(2) (F^-) = 0.05 ppm. To 5 ml stock solution C, add 20 ml TISAB and dilute to 100 ml with distilled water.

(3) (F^-) = 0.10 ppm. To 10 ml stock solution C, add 20 ml TISAB and dilute to 100 ml with distilled water.

(4) (F^-) = 0.50 ppm. To 50 ml stock solution C, add 20 ml TISAB and dilute to 100 ml with distilled water.

(5) (F^-) = 1.00 ppm. To 10 ml stock solution B, add 20 ml TISAB and dilute to 100 ml with distilled water.

(6) (F^-) = 10.00 ppm. To 1 ml stock solution A, add 20 ml TISAB and dilute to 100 ml with distilled water.

Note: Higher concentrated standards may be prepared if necessary. Linear range for millivolt readings extends beyond, but not below, concentrations described above.

6.012D. Preparation of Standard Curve.

(a) According to manufacturer's instructions, verify that pH/mv meter reads from -220 to $+220$ mv.

(b) Place electrodes in 100 ml polypropylene beaker containing 10.00 ppm working standard on a magnetic stirrer, and stir. Set the meter function switch to read expanded millivolts and turn calibration control to read 000.0 mv.

(c) Rinse electrodes, blot dry, and place in 1.00 ppm F^- standard. Stirring continuously, wait for reading to stabilize. Stabilization is indicated by no more than 0.5 mv change over a 5 min period. Waiting period increases with decreasing concentration.

(d) Repeat step C, above, with rest of the standards in order of decreasing concentration.

(e) On standard 2-cycle semi-log paper, plot mv versus concentration, with mv on the horizontal axis and concentration on the vertical (log) axis. Three-cycle paper can be used if higher concentrated standards are required.

6.012E. Determination

(a) Weigh 10 g sample into 16 oz polyethylene bottle.

(b) Run a tissue blank and a recovery in parallel with sample(s). Add 10.0 ml of 10.0 ppm F^- standard solution to 10 g blank tissue for 10 ppm F^- fortification.

(c) Extract fat twice with petroleum ether as follows: Add 100 ml petroleum ether to each sample, blank, and recovery. Break up lumps with plastic stirring rod. Stopper with silicone stopper and shake vigorously for 30 seconds. Centrifuge for 10 min. at 2,500 rpm. Decant and discard ether layer, being careful not to include any aqueous phase. Repeat extraction.

(d) Place sample bottles in water bath and carefully evaporate any residual ether by gradually raising the bath temperature to 100°C to prevent spattering.

(e) Remove samples immediately, cool, and add 50 ml TISAB solution and 50 ml 5% disodium EDTA. Mix with Tissumizer until homogenous.

(f) Place sample bottles in covered constant temperature water bath that has attained nearly 100°C . (Salt may be added to bath to increase boiling point.)

(g) Place thermometer into one sample bottle until temperature of sample reaches 90°C . Remove cover and leave samples in bath for 1 hr. (Samples will reach an equilibrium temperature of approximately 94°C .) Remove samples and let cool.

- (h) Break up lumps with plastic stirring rod.
- (i) Transfer samples to 250 ml graduated cylinders. Rinse bottle several times into cylinder and dilute samples to 250 ml with distilled water.
- (j) Stopper and shake thoroughly.
- (k) Filter sample solutions through fluted filter paper into polyethylene bottles. Do not use glass bottles.
- (l) Pour about 100 ml sample solution into polypropylene beaker and with constant stirring, insert electrodes and record mv reading at equilibrium.

6.012F. Calculations

Compare mv sample reading to standard curve to obtain ppm F^- in sample solution.

$$\text{ppm } F^- \text{ in sample} = \frac{A B}{C}$$

where: A = ppm F^- in sample solution
B = ml final dilution
C = g sample

$$\text{If procedure is followed as written, ppm } F^- = \frac{250A}{10} = 25A$$

Note: Specific ion and pH meters equipped with direct concentration readout via microprocessor may be used.

Ref: Dolan, et al: J. Assoc. Off. Anal. Chem. (Vol. 61, No. 4, 1978, p. 982).

APPENDIX

I. Rounding-Off Numbers and Significant Figures

Most analytical results calculated from the methods in this Guidebook carry one, two, three, or more decimal places. Results will be rounded off to the nearest tenth and any digits beyond the tenth place will be dropped.

In order to avoid problems with data processing, it is imperative that decimals be rounded in the same manner. The following procedure has been determined to be best:

When the first or succeeding digit dropped is less than 5, the preceding digit is not changed. When the succeeding digit is 5 or more, the preceding digit is increased by 1. Some examples are:

<i>Number</i>	<i>Rounded Value</i>
0.850	0.9
0.751	0.8
0.749	0.7
3.54	3.5
5.949	5.9

Webster's Third New International Dictionary defines significant figures as follows:

"Figures of a number that begin with the first figure to the left that is not zero and that end with the last figure to the right that is not zero or is a zero that is considered to be correct"

Therefore:

1.0101	=	5 significant figures
1.01010	=	6 significant figures
0.0101	=	3 significant figures
0.01010	=	4 significant figures

IA. Rules for Reporting Analytical Results for Validated Methods

1. All values are corrected for recovery using the running average of the last 10 acceptable recoveries (\bar{R}). If a recovery is not acceptable, the sample or set of samples run with that recovery are reanalyzed. Rule 5, rounding, is applied as the final step in calculating a value for reporting.

Example: Acceptable Recovery Range for CHC analysis = 80 – 110%
Standard Deviation Repeatability = (± 0.012)

Last 10 recoveries: 84%

87

82

89

92

95

88

85

90

80

Running Average Recovery = \bar{R} = 88%

Analytical Finding = 0.138

Recovery Corrected Value = $0.138 \times 100/88 = 0.157 = 0.16$

2. Levels with instrumental responses not exceeding three times the noise level (electronic chatter or background aberrations) are reported as not detected (ND).

3. Levels above 3 times the noise level are reported as the rounded, recovery corrected value.

Example: Average Recovery = 95%

Analytical Finding = 0.41 ppm

Reported Value = $0.41 \times 100/95 = 0.43$ ppm

4. The first non-zero digit of the standard deviations for repeatability of the analytical method will determine the number of significant digits for reporting, up to an analytical value of 100 units. At or above 100 units, the reported value is the rounded whole number value. Rounding is done as described in "Guidebook" Appendix I, page A-1.

Examples:

<u>Repeatability Standard Deviation</u>	<u>Analytical Finding</u>	<u>Report</u>	<u>Analytical Finding</u>	<u>Report</u>
$\pm 0.01 - \pm 0.09$	0.108	0.11	0.104	0.10
	1.08	1.08	1.04	1.04
	10.8	10.8	10.4	10.4
	108	108	104	104
	1080	1080	1040	1040
$\pm 0.10 - \pm 0.99$	0.108	0.1	0.104	0.1
	1.08	1.1	1.04	1
	10.8	10.8	10.4	10.4
	108	108	104	104
	1080	1080	1040	1040
≥ 1.0	0.108	*	0.104	*
	1.08	1	1.04	1
	10.8	11	10.4	10
	108	108	104	104
	1080	1080	1040	1040

* Apply rule 1, 2, 3, or 4, as appropriate.

II A.—Protein Multipliers

3.79 Protein Multiplier

%	Product	%	Product	%	Product	%	Product	%	Product
8.0	30.3	11.0	41.7	14.0	53.1	17.0	64.4	20.0	75.8
8.1	30.7	11.1	42.1	14.1	53.4	17.1	64.8	20.1	76.2
8.2	31.1	11.2	42.4	14.2	53.8	17.2	65.2	20.2	76.6
8.3	31.5	11.3	42.8	14.3	54.2	17.3	65.6	20.3	76.9
8.4	31.8	11.4	43.2	14.4	54.6	17.4	65.9	20.4	77.3
8.5	32.2	11.5	43.6	14.5	55.0	17.5	66.3	20.5	77.7
8.6	32.6	11.6	44.0	14.6	55.3	17.6	66.7	20.6	78.1
8.7	33.0	11.7	44.3	14.7	55.7	17.7	67.1	20.7	78.5
8.8	33.4	11.8	44.7	14.8	56.1	17.8	67.5	20.8	78.8
8.9	33.7	11.9	45.1	14.9	56.5	17.9	67.8	20.9	79.2
9.0	34.1	12.0	45.5	15.0	56.8	18.0	68.2	21.0	79.6
9.1	34.5	12.1	45.9	15.1	57.2	18.1	68.6	21.1	80.0
9.2	34.9	12.2	46.2	15.2	57.6	18.2	69.0	21.2	80.3
9.3	35.2	12.3	46.6	15.3	58.0	18.3	69.4	21.3	80.7
9.4	35.6	12.4	47.0	15.4	58.4	18.4	69.7	21.4	81.1
9.5	36.0	12.5	47.4	15.5	58.7	18.5	70.1	21.5	81.5
9.6	36.4	12.6	47.8	15.6	59.1	18.6	70.5	21.6	81.9
9.7	36.8	12.7	48.1	15.7	59.5	18.7	70.9	21.7	82.2
9.8	37.1	12.8	48.5	15.8	59.9	18.8	71.3	21.8	82.6
9.9	37.5	12.9	48.9	15.9	60.3	18.9	71.6	21.9	83.0
10.0	37.9	13.0	49.3	16.0	60.6	19.0	72.0	22.0	83.4
10.1	38.3	13.1	49.6	16.1	61.0	19.1	72.4	22.1	83.8
10.2	38.7	13.2	50.0	16.2	61.4	19.2	72.8	22.2	84.1
10.3	39.0	13.3	50.4	16.3	61.8	19.3	73.1	22.3	84.5
10.4	39.4	13.4	50.8	16.4	62.2	19.4	73.5	22.4	84.9
10.5	39.8	13.5	51.2	16.5	62.5	19.5	73.9	22.5	85.3
10.6	40.2	13.6	51.5	16.6	62.9	19.6	74.3	22.6	85.7
10.7	40.6	13.7	51.9	16.7	63.3	19.7	74.7	22.7	86.0
10.8	40.9	13.8	52.3	16.8	63.7	19.8	75.0	22.8	86.4
10.9	41.3	13.9	52.7	16.9	64.1	19.9	75.4	22.9	86.8

II B.—Protein Multipliers

3.8 Protein Multiplier

%	Product	%	Product	%	Product	%	Product	%	Product
8.0	30.4	11.0	41.8	14.0	53.2	17.0	64.6	20.0	76.0
8.1	30.8	11.1	42.2	14.1	53.6	17.1	65.0	20.1	76.4
8.2	31.2	11.2	42.6	14.2	54.0	17.2	65.4	20.2	76.8
8.3	31.5	11.3	42.9	14.3	54.3	17.3	65.7	20.3	77.1
8.4	31.9	11.4	43.3	14.4	54.7	17.4	66.1	20.4	77.5
8.5	32.3	11.5	43.7	14.5	55.1	17.5	66.5	20.5	77.9
8.6	32.7	11.6	44.1	14.6	55.5	17.6	66.9	20.6	78.3
8.7	33.1	11.7	44.5	14.7	55.9	17.7	67.3	20.7	78.7
8.8	33.4	11.8	44.8	14.8	56.2	17.8	67.6	20.8	79.0
8.9	33.8	11.9	45.2	14.9	56.6	17.9	68.0	20.9	79.4
9.0	34.2	12.0	45.6	15.0	57.0	18.0	68.4	21.0	79.8
9.1	34.6	12.1	46.0	15.1	57.4	18.1	68.8	21.1	80.2
9.2	35.0	12.2	46.4	15.2	57.8	18.2	69.2	21.2	80.6
9.3	35.3	12.3	46.7	15.3	58.1	18.3	69.5	21.3	80.9
9.4	35.7	12.4	47.1	15.4	58.5	18.4	69.9	21.4	81.3
9.5	36.1	12.5	47.5	15.5	58.9	18.5	70.3	21.5	81.7
9.6	36.5	12.6	47.9	15.6	59.3	18.6	70.7	21.6	82.1
9.7	36.9	12.7	48.3	15.7	59.7	18.7	71.1	21.7	82.5
9.8	37.2	12.8	48.6	15.8	60.0	18.8	71.4	21.8	82.8
9.9	37.6	12.9	49.0	15.9	60.4	18.9	71.8	21.9	83.2
10.0	38.0	13.0	49.4	16.0	60.8	19.0	72.2	22.0	83.6
10.1	38.4	13.1	49.8	16.1	61.2	19.1	72.6	22.1	84.0
10.2	38.8	13.2	50.2	16.2	61.6	19.2	73.0	22.2	84.4
10.3	39.1	13.3	50.5	16.3	61.9	19.3	73.3	22.3	84.7
10.4	39.5	13.4	50.9	16.4	62.3	19.4	73.7	22.4	85.1
10.5	39.9	13.5	51.3	16.5	62.7	19.5	74.1	22.5	85.5
10.6	40.3	13.6	51.7	16.6	63.1	19.6	74.5	22.6	85.9
10.7	40.7	13.7	52.1	16.7	63.5	19.7	74.9	22.7	86.3
10.8	41.0	13.8	52.4	16.8	63.8	19.8	75.2	22.8	86.6
10.9	41.4	13.9	52.8	16.9	64.2	19.9	75.6	22.9	87.0

II C.—Protein Multipliers

3.83 Protein Multiplier

%	Product	%	Product	%	Product	%	Product	%	Product
8.0	30.6	11.0	42.1	14.0	53.6	17.0	65.1	20.0	76.6
8.1	31.0	11.1	42.5	14.1	54.0	17.1	65.5	20.1	77.0
8.2	31.4	11.2	42.9	14.2	54.4	17.2	65.9	20.2	77.4
8.3	31.8	11.3	43.3	14.3	54.8	17.3	66.3	20.3	77.7
8.4	32.2	11.4	43.7	14.4	55.2	17.4	66.6	20.4	78.1
8.5	32.6	11.5	44.0	14.5	55.5	17.5	67.0	20.5	78.5
8.6	32.9	11.6	44.4	14.6	55.9	17.6	67.4	20.6	78.9
8.7	33.3	11.7	44.8	14.7	56.3	17.7	67.8	20.7	79.3
8.8	33.7	11.8	45.2	14.8	56.7	17.8	68.2	20.8	79.7
8.9	34.1	11.9	45.6	14.9	57.1	17.9	68.6	20.9	80.0
9.0	34.5	12.0	46.0	15.0	57.4	18.0	68.9	21.0	80.4
9.1	34.9	12.1	46.3	15.1	57.8	18.1	69.3	21.1	80.8
9.2	35.2	12.2	46.7	15.2	58.2	18.2	69.7	21.2	81.2
9.3	35.6	12.3	47.1	15.3	58.6	18.3	70.1	21.3	81.6
9.4	36.0	12.4	47.5	15.4	59.0	18.4	70.5	21.4	82.0
9.5	36.4	12.5	47.9	15.5	59.4	18.5	70.9	21.5	82.3
9.6	36.8	12.6	48.3	15.6	59.7	18.6	71.2	21.6	82.7
9.7	37.2	12.7	48.6	15.7	60.1	18.7	71.6	21.7	83.1
9.8	37.5	12.8	49.0	15.8	60.5	18.8	72.0	21.8	83.5
9.9	37.9	12.9	49.4	15.9	60.9	18.9	72.4	21.9	83.9
10.0	38.3	13.0	49.8	16.0	61.3	19.0	72.8	22.0	84.3
10.1	38.7	13.1	50.2	16.1	61.7	19.1	73.2	22.1	84.6
10.2	39.1	13.2	50.6	16.2	62.0	19.2	73.5	22.2	85.0
10.3	39.4	13.3	50.9	16.3	62.4	19.3	73.6	22.3	85.4
10.4	39.8	13.4	51.3	16.4	62.8	19.4	74.3	22.4	85.8
10.5	40.2	13.5	51.7	16.5	63.2	19.5	74.7	22.5	86.2
10.6	40.6	13.6	52.1	16.6	63.6	19.6	75.1	22.6	86.6
10.7	41.0	13.7	52.5	16.7	64.0	19.7	75.5	22.7	86.9
10.8	41.4	13.8	52.9	16.8	64.3	19.8	75.8	22.8	87.3
10.9	41.7	13.9	53.2	16.9	64.7	19.9	76.2	22.9	87.7

II D.—Protein Multipliers

3.93 Protein Multiplier

%	Product	%	Product	%	Product	%	Product	%	Product
8.0	31.4	11.0	43.2	14.0	55.0	17.0	66.8	20.0	78.6
8.1	31.8	11.1	43.6	14.1	55.4	17.1	67.2	20.1	79.0
8.2	32.2	11.2	44.0	14.2	55.8	17.2	67.2	20.2	79.4
8.3	32.6	11.3	44.4	14.3	56.2	17.3	68.0	20.3	79.8
8.4	33.0	11.4	44.8	14.4	56.6	17.4	68.4	20.4	80.2
8.5	33.4	11.5	45.2	14.5	57.0	17.5	68.8	20.5	80.6
8.6	33.8	11.6	45.6	14.6	57.4	17.6	69.2	20.6	81.0
8.7	34.2	11.7	46.0	14.7	57.8	17.7	69.6	20.7	81.4
8.8	34.6	11.8	46.4	14.8	58.2	17.8	70.0	20.8	81.7
8.9	35.0	11.9	46.8	14.9	58.6	17.9	70.3	20.9	82.1
9.0	35.4	12.0	47.2	15.0	59.0	18.0	70.7	21.0	82.5
9.1	35.8	12.1	47.6	15.1	59.3	18.1	71.1	21.1	82.9
9.2	36.2	12.2	47.9	15.2	59.7	18.2	71.5	21.2	83.3
9.3	36.5	12.3	48.3	15.3	60.1	18.3	71.9	21.3	83.7
9.4	36.9	12.4	48.7	15.4	60.5	18.4	72.3	21.4	84.1
9.5	37.3	12.5	49.1	15.5	60.9	18.5	72.7	21.5	84.5
9.6	37.7	12.6	49.5	15.6	61.3	18.6	73.1	21.6	84.9
9.7	38.1	12.7	49.9	15.7	61.7	18.7	73.5	21.7	85.3
9.8	38.5	12.8	50.3	15.8	62.1	18.8	73.9	21.8	85.7
9.9	38.9	12.9	50.7	15.9	62.5	18.9	74.3	21.9	86.1
10.0	39.3	13.0	51.1	16.0	62.9	19.0	74.7	22.0	86.5
10.1	39.7	13.1	51.5	16.1	63.3	19.1	75.1	22.1	86.9
10.2	40.1	13.2	51.9	16.2	63.7	19.2	75.5	22.2	87.2
10.3	40.5	13.3	52.3	16.3	64.1	19.3	75.8	22.3	87.6
10.4	40.9	13.4	52.7	16.4	64.5	19.4	76.2	22.4	88.0
10.5	41.3	13.5	53.1	16.5	64.8	19.5	76.6	22.5	88.4
10.6	41.7	13.6	53.4	16.6	65.2	19.6	77.0	22.6	88.8
10.7	42.1	13.7	53.8	16.7	65.6	19.7	77.4	22.7	89.2
10.8	42.4	13.8	54.2	16.8	66.0	19.8	77.8	22.8	89.6
10.9	42.8	13.9	54.6	16.9	66.4	19.9	78.2	22.9	90.0

III.—Conversion Table “Fractions to Decimals”

$\frac{1}{64} = .015625$	$\frac{17}{64} = .265625$	$\frac{33}{64} = .515625$	$\frac{49}{64} = .765625$
$\frac{1}{32} = .03125$	$\frac{9}{32} = .28125$	$\frac{17}{32} = .53125$	$\frac{25}{32} = .78125$
$\frac{3}{64} = .046875$	$\frac{19}{64} = .296875$	$\frac{35}{64} = .546875$	$\frac{51}{64} = .796875$
$\frac{1}{16} = .0625$	$\frac{5}{16} = .3125$	$\frac{9}{16} = .5625$	$\frac{13}{16} = .8125$
$\frac{5}{64} = .078125$	$\frac{21}{64} = .328125$	$\frac{37}{64} = .578125$	$\frac{53}{64} = .828125$
$\frac{3}{32} = .09375$	$\frac{11}{32} = .34375$	$\frac{19}{32} = .59375$	$\frac{27}{32} = .84375$
$\frac{7}{64} = .109375$	$\frac{23}{64} = .359375$	$\frac{39}{64} = .609375$	$\frac{55}{64} = .859375$
$\frac{1}{8} = .125$	$\frac{3}{8} = .375$	$\frac{5}{8} = .625$	$\frac{7}{8} = .875$
$\frac{9}{64} = .140625$	$\frac{25}{64} = .390625$	$\frac{41}{64} = .640625$	$\frac{57}{64} = .890625$
$\frac{5}{32} = .15625$	$\frac{13}{32} = .40625$	$\frac{21}{32} = .65625$	$\frac{29}{32} = .90625$
$\frac{11}{64} = .171875$	$\frac{27}{64} = .421875$	$\frac{43}{64} = .671875$	$\frac{59}{64} = .921875$
$\frac{3}{16} = .1875$	$\frac{7}{16} = .4375$	$\frac{11}{16} = .6875$	$\frac{15}{16} = .9375$
$\frac{13}{64} = .203125$	$\frac{29}{64} = .453125$	$\frac{45}{64} = .703125$	$\frac{61}{64} = .953125$
$\frac{7}{32} = .21875$	$\frac{15}{32} = .46875$	$\frac{23}{32} = .71875$	$\frac{31}{32} = .96875$
$\frac{15}{64} = .234375$	$\frac{31}{64} = .484375$	$\frac{47}{64} = .734375$	$\frac{63}{64} = .984375$
$\frac{1}{4} = .25$	$\frac{1}{2} = .5$	$\frac{3}{4} = .75$	$1 = 1.0$

IV.—Conversion Table “Avoirdupois to Metric”

<i>Pounds — Grams</i>		<i>Ounces — Grams</i>		<i>Ounces — Grams</i>	
1	453.6	1	28.4	1/16	1.77
2	907.2	2	56.7	1/8	3.54
3	1360.8	3	85.1	3/16	5.32
4	1814.4	4	113.4	1/4	7.09
5	2268.0	5	141.8	5/16	8.56
6	2721.5	6	170.1	3/8	10.6
7	3175.1	7	198.4	7/16	12.4
8	3628.7	8	226.8	1/2	14.2
9	4082.3	9	255.2	9/16	16.0
10	4535.9	10	283.5	5/8	17.7
11	4989.5	11	311.9	11/16	19.5
12	5443.1	12	340.2	3/4	21.3
		13	368.6	13/16	23.0
		14	396.9	7/8	24.8
		15	425.3	15/16	26.6
		16	453.6	1	28.4

V.—Table of Four—Place Logarithms

Proportional Parts											Proportional Parts																			
0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9											
0000	0043	0086	0128	0170	0212	0253	0294	0334	0371	55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	3	4	5	6	7	8	9	
0111	0153	0192	0231	0269	0307	0345	0382	0419	0455	56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	3	4	5	6	7	8	9	
0202	0242	0280	0317	0354	0390	0426	0461	0496	0531	57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	3	4	5	6	7	8	9	
0292	0331	0368	0404	0439	0474	0508	0542	0576	0609	58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	5	6	7	8	9
0382	0419	0455	0490	0524	0558	0591	0624	0657	0689	59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	5	6	7	8	9
0471	0507	0542	0576	0609	0642	0674	0706	0737	0768	60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	5	6	7	8	9
0560	0595	0628	0660	0691	0722	0752	0781	0811	0840	61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	5	6	7	8	9
0648	0682	0714	0745	0775	0804	0833	0861	0889	0917	62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	4	5	6	7	8	9
0736	0769	0800	0830	0858	0886	0913	0940	0966	0992	63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	4	5	6	7	8	9
0824	0856	0886	0915	0943	0970	0996	1022	1048	1073	64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	4	5	6	7	8	9
0911	0942	0971	1000	1027	1054	1080	1106	1131	1156	65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	4	5	6	7	8	9
1000	1029	1057	1084	1110	1135	1160	1185	1210	1234	66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	4	5	6	7	8	9
1087	1115	1142	1168	1193	1218	1242	1266	1290	1314	67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	4	5	6	7	8	9
1175	1202	1228	1253	1277	1301	1325	1348	1371	1394	68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	4	5	6	7	8	9
1262	1288	1313	1337	1360	1383	1405	1427	1449	1471	69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	3	4	5	6	7	8	9
1350	1375	1398	1421	1443	1465	1486	1508	1529	1549	70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	3	4	5	6	7	8	9
1437	1461	1484	1506	1527	1548	1568	1588	1608	1627	71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	3	4	5	6	7	8	9
1515	1538	1560	1581	1602	1622	1642	1661	1680	1699	72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	3	4	5	6	7	8	9
1603	1625	1646	1666	1686	1705	1724	1743	1761	1779	73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8689	1	1	2	3	4	5	6	7	8	9
1691	1712	1732	1751	1770	1788	1806	1824	1842	1859	74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	3	4	5	6	7	8	9
1747	1767	1786	1804	1822	1839	1856	1873	1889	1906	75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	3	4	5	6	7	8	9
1835	1853	1870	1887	1903	1919	1935	1950	1966	1981	76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	3	4	5	6	7	8	9
1920	1937	1953	1968	1983	1998	2013	2027	2042	2056	77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	3	4	5	6	7	8	9
2007	2022	2037	2051	2065	2079	2093	2107	2120	2134	78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	3	4	5	6	7	8	9
2080	2094	2107	2120	2133	2145	2157	2169	2181	2193	79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9026	1	1	2	3	4	5	6	7	8	9
2146	2158	2170	2181	2192	2203	2214	2225	2235	2245	80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	3	4	5	6	7	8	9
2197	2208	2218	2228	2238	2247	2257	2266	2275	2284	81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	3	4	5	6	7	8	9
2235	2245	2254	2263	2272	2281	2290	2299	2308	2316	82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	3	4	5	6	7	8	9
2266	2275	2283	2292	2300	2308	2316	2324	2332	2340	83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	3	4	5	6	7	8	9
2299	2311	2319	2326	2334	2341	2348	2355	2362	2369	84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	3	4	5	6	7	8	9
2339	2349	2354	2359	2364	2369	2374	2378	2383	2387	85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	3	4	5	6	7	8	9
2369	2372	2375	2378	2381	2384	2387	2390	2393	2396	86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	3	4	5	6	7	8	9
2379	2381	2384	2386	2388	2390	2392	2394	2396	2398	87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	2	3	4	5	6	7	8	9
2389	2390	2392	2394	2395	2396	2397	2398	2399	2400	88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	2	3	4	5	6	7	8	9
2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	2	3	4	5	6	7	8	9
2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	2	3	4	5	6	7	8	9
2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	2	3	4	5	6	7	8	9
2429	2430	2431	2432	2433	2434	2435	2436	2437	2438	92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	2	3	4	5	6	7	8	9
2439	2440	2441	2442	2443	2444	2445	2446	2447	2448	93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	2	3	4	5	6	7	8	9
2449	2450	2451	2452	2453	2454	2455	2456	2457	2458	94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	2	3	4	5	6	7	8	9
2459	2460	2461	2462	2463	2464	2465	2466	2467	2468	95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	2	3	4	5	6	7	8	9
2469	2470	2471	2472	2473	2474	2475	2476	2477	2478	96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	2	3	4	5	6	7	8	9
2479	2480	2481	2482	2483	2484	2485	2486	2487	2488	97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	2	3	4	5	6	7	8	9
2489	2490	2491	2492	2493	2494	2495	2496	2497	2498	98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	2	3	4	5	6	7	8	9
2499	2500	2501	2502	2503	2504	2505	2506	2507	2508	99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	2	3	4	5	6	7	8	9

VI — Water Protein Ratios
Interim Subject to Revision

<i>Organs</i>	<i>Average</i>
Beef Tongue	4.4/1
Pork Tongue	3.9/1
Beef Hearts	4.4/1
Pork Hearts	4.8/1
Beef Tripe (raw)	5.2/1
Beef Tripe (scalded)	6.3/1
Beef Tripe (cooked)	5.1/1
Pork Stomach	4.2/1

Maximum Expected of Products

Basturma	2.04/1	Milan Sausage	1.9/1
Beef (dried)	2.04/1	Milano Sausage	1.9/1
(air dried)	2.04/1	Mortadella (canned)	3.85/1
(slice dried)	2.04/1	(poultry, canned)	3.85/1
Beef Bouillon	67/1	Parma Ham (Ham-Parma)	2.03/1
Beef Broth	135/1	Pepperoni	1.6/1
Beef Stock	135/1	Pepper Sticks	1.9/1
Blockwurst	3.7/1	Poultry Franks	No requirements
Brown and Serve Sausage	3.7/1	Roast Beef (canned)	2.25/1
Cervelat	No requirement	(cooked)	3.8/1
Chipped Beef (chunked)	2.04/1	Salami (dry)	1.9/1
(ground)	2.04/1	(dry, NFDM added)	1.9/1
(chopped and formed)	2.04/1	(German Brand)	1.9/1
(chunked and formed)	2.04/1	(Italian)	1.9/1
Chirizo	1.9/1	(Turkey)	1.9/1
Chirizo in Lard (canned)	1.8/1	(Cooked Turkey)	1.9/1
Chirizo (Shelf Stable, vacuum packed)	3.1/1	Salted Beef	2/1
Condensed Meat Broth	67/1	Sausage Sticks	1.9/1
Condensed Meat Stock	67/1	Self Basting Beef for Roasting	3.8/1
Cooked Beef Roast	3.8/1	Sicilian Salami	2.3/1
Cooked Salami	1.9/1	Sliced Dried Beef in Jars	2.04/1
Corned Beef (canned)	2.28/1	Soppresate	1.9/1
Corned Mutton (canned)	2.28/1	Soup (condensed)	67/1
Dried Meat	2.04/1	(Ready to Eat)	135/1
Dry Sausage	1.9/1	Summer Sausage (Shelf Stable)	3.1/1
Farmer Summer Sausage	1.9/1	Summer Sausage (Keep Refrigerated)	3.7/1
Frizzes	1.6/1	Tasajo	2/1
Genoa Salami	2.3/1	Thuringer	3.7/1
Hunters Sausage	4/1	Touristen Wurst	3.7/1
Jerkey (beef)	0.75/1	Tropi-Cure Pork Products	3.25/1
(natural)	0.75/1	Turkey Pepperoni	1.6/1
(turkey)	0.75/1	Ukarianian Sausage	2/1
Kipperd Beef	2.03/1	Veal	4/1
Landjaeger	1.9/1		
Meat Broth	135/1		
Meat Stock	135/1		

VII.—Ascorbate Levels in Spices

When seasoning mixtures are analyzed for undeclared ascorbates and erythorbates, an allowance should be made for naturally occurring ene-diol type compounds. Listed below are average values of the ene-diol content (calculated as sodium erythorbate) of some common ingredients of seasoning mixtures:

<i>Ingredient</i>	<i>ppm Sodium Erythorbate</i>
Ground White Pepper	100
Brown Sugar	280
Onion Powder	280
Dextrose	70
Coriander	100
Milled Mustard	300
Ginger	120
Dendritic Salt	7
Paprika	500 (may assay as high as 800 ppm, depending upon origin)

Mixtures containing some or all of these ingredients should not, of course, contain a concentration of ascorbate or erythorbate greater than that found in the highest bearing components.

Samples for which a specific request for ascorbates or erythorbates has been made and which do not materially exceed the above values should be reported as "containing no excessive ascorbate (or erythorbate)."